An Investigation of the Interactions of DVAP-33A, the Orthologue of Human VAPB.

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Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

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Abstract

Amyotrophic Lateral Sclerosis is the most common type of motor neuron disease, characterized by progressive degeneration of the upper and lower motor neurons. Sufferers present with symptoms of muscle weakness and this quickly develops on to paralysis and finally death due to respiratory failure within 5 years of disease onset. Although the majority of cases are sporadic, about 10% are familial and it is hoped that through the investigation of these few cases a greater understanding of the disease process, the reasons for its delayed onset and vulnerability of motor neurons will be achieved. Recently a novel mutation linked to ALS was discovered in an evolutionary conserved protein named Vesicle associated membrane protein (VAMP) associated protein B (VAPB). VAPB is an integral type II membrane protein localised at the Endoplasmic Reticulum and thought to have a role in protein transport. The orthologue in *Drosophila* has been shown to be involved in the homeostatic regulation of bouton formation at the Neuromuscular Junction through an association with the microtubule network. To elucidate the mechanism through which this protein causes ALS, Pennetta *et al* have created a *Drosophila* model of the disease by expressing the mutated orthologue in the fly. To complement this model, I have undertaken a number of biochemical experiments to look for potential interactors of the VAP proteins. The yeast two hybrid system utilises the yeast GAL4 transcriptional activator to indicate a protein interaction within a yeast cell and can be used to test a cDNA library for interactors. Through this technique a number of interesting binding partners have been found that may play crucial roles in the progression of the disease.

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Abbreviations

ATF6	Activating transcription factor 6
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
BMAA	β- methylaminoalanine
CC	Coiled coil
Cdc42	Cell division cycle 42
CERT	Ceramide transfer protein
CMT	Charcot-Marie-Tooth
CPE	Ceramide phospoethanolamine
CSPα	Cysteine String Protein Alpha
CST	Corticospinal tract
Cy3	Cyanine
DAG	Diacylglycerol
ddH20	Double distilled water
Dbl	Diffuse B-cell lymphoma
DNA	Deoxyribonucleic acid
DVAP-33	*Drosophila* VAMP associated protein of 33kDa
ER	Endoplasmic reticulum
ERAD	Endoplasmic associated degradation
FA	Fatty Acid
FITC	Fluorescein isothiocyanate
FTLD	Frontotemporal lobar degeneration
FUS/TLS	Fused in sarcoma/translocated in liposarcoma
GEF	Guanine nucleotide exchange factor
GFP	Green Fluorescent Protein
Gmr	Glass multiple reporter
GS	Glucan synthase
HD	Huntington's Disease

HRP	Horseradish peroxidase
HSP70	Heat shock protein of 70kDa
HVAPB	Human VAMP associated protein B
IRE1	Inositol requiring enzyme 1
LOH	Loss of heterozygosity
MAP	Microtubule associated protein
mEJPS	Minature excitatory junctional potentials
Min	Minutes
MND	Motor Neuron Disease
MSP	Major sperm protein
NGS	Normal Goat Serum
NMJ	Neuromuscular junction
25OH	25-Hydroxycholesterol
ORF	Open reading frame
OSBP	Oxysterol-binding protein
P56S	Proline to Serine substitution
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBT	Phosphate buffer saline with Triton
PD	Parkinson's Disease
PDI	Protein disulphide isomerase
PERK	Protein kinase-like ER kinase
PH	pleckstrin homology
PI	Phosphoinositide
PIP	Phosphoinositol phosphate
RdgB	Retinal degradation B
RNA	Ribonucleic acid
RRM	RNA recognition motif
SAC1	Suppressor of Actin 1
SCA 8	Spinocerebellar Ataxia 8

SEM	Scanning Electron Microscopy
SETX	Senataxin
SM	Sphingomyelin
SMA	Spinal muscular atrophy
SOD1	Superoxide Dismutase 1
TAG	Triacylglyceride
TBS	Tris Buffer Solution
TBST	Tris buffer with Tween
TDP	Transactive Response (TAR) -DNA binding protein
TEM	Transmission Electron Microscopy
TRN	Trans Golgi network
TMD	Transmembrane domain
UAS	Upstream Activating Sequence
UBI	Ubiquitinated Inclusion
UPR	Unfolded protein response
VAMP	Vesicle Associated Protein (Synaptobrevin)
Wt	Wild Type
yw	Yellow white
ml	millilitre
µm	micron
µl	microlitre

Chapter 1: Introduction

1.1 Neurodegenerative disease

Neurodegeneration is a feature of many devastating diseases and not surprisingly much time and research has been spent trying to elucidate the underlying pathomechanisms. Unfortunately however, although some major progress has been made there is still little known about the pathogenesis of many degenerative diseases. In particular, there is no solid understanding of why only certain subsets of neurons are vulnerable in the various diseases, for example, motor neurons in motor neuron disease (MND) and the dopaminergic neurons of the substantia nigra in Parkinson's disease (Bonini and Fortini 2003; Bruijn *et al.* 2004) . There is also much debate as to whether malfunction and degeneration of the cell body, axon or synapse is the primary pathological event (Pun *et al.* 2006). The focus of the work in the Pennetta lab addresses the motor neuron disease Amyotrophic Lateral Sclerosis (ALS). We use *Drosophila melanogaster* as a model system for this disease and harness its genetic potential as a tool to examine the contribution of various cellular mechanisms to the onset and progression of disease. Synaptic homeostasis and dysfunction, mRNA regulation, transcriptional regulation, chaperone and stress pathways, and the proteosome pathway have all been implicated in disease and are all vital areas of investigation.

1.2 Bigger is not always better when modeling disease

Drosophila has many advantages over larger model organisms as although it has a relatively simple structure, many of the basic components are very similar including mechanisms and molecular pathways involved in human disease (St Johnston 2002; Bonini and Fortini 2003). The same is true for other simple organisms including the yeast *Saccharomyces cerevisiae* which has also been used to investigate a number of key aspects of disease including Huntington's Disease (HD) and Parkinson's Disease (PD). A major hallmark of many degenerative diseases is the presence of protein aggregates and the cellular response to mis-folded proteins is largely conserved from yeast to man.

Outeiro *et al* (2003) created a fusion protein of the green flurescent protein (GFP) with α-synuclein; a protein linked to familial PD and found in the cytoplasmic inclusions (Lewy bodies) that are a hallmark of disease. By doubling the expression of α-synuclein in the cell, the protein was observed to relocate away from the plasma membranes and form cytoplasmic inclusions and the growth of yeast expressing this fusion protein was inhibited (Outeiro and Lindquist 2003). By expressing 5000 individual genes in combination with α-synuclein, Gitler *et al* (2008) screened for genes that modified the toxic properties of α-synuclein leading to the discovery of Ypt1p (Gitler 2008). When over-expressed in combination with α-synuclein, Ypt1p allowed a more robust growth of yeast and as such was proposed to suppress the toxicity of α-synuclein. When the mammalian orthologue Rab1 was tested in a range of neuronal models including *Drosophila* and *C. elegans,* a suppression of dopaminergic degeneration was seen, adding validity to the original screen in yeast (Cooper *et al.* 2006; Gitler *et al.* 2008).

Screens such as those described here by Gitler *et al* (2008) are a good example of how the simple yeast cell can be a powerful tool for carrying out large scale screens for potential therapeutic targets and genetic modifiers of disease. This is further shown in a screen for HD, a disease caused by the expansion of a CAG repeat in the gene *IT-15* which encodes huntingtin. (Muchowski *et al.* 2002; Willingham *et al.* 2003) Expression of the expanded *IT-15* in yeast cells caused an accumulation of the expanded huntingtin protein (HD53Q) and the formation of aggregates but with no effect on viability. A screen was thus carried out to find non-lethal gene mutations that when combined with huntingtin, enhanced the phenotype so that the combination was toxic to the cells. Mutations in chaperone proteins and other genes involved in the cellular stress response were found to increase the toxicity of HD53Q (Willingham *et al.* 2003). This provides a direction for the focus of research in larger more complex systems.

No system can be perfect for modeling disease and the major drawback of the yeast cell is that it is a single celled organism so research into the more complex aspects of disease such as why it only affects subsets of neurons is not possible. Perhaps the next step is to use cell culture as a model. Here it can be possible to grow the specific cells affected by disease e.g. motor neurons, in a carefully controlled environment. The

effect of a number of variables can be tested on the cells and observed more easily than in the dissection of an organism that would be necessary *in vivo.* Cell culture has been vitally important in modeling disease and particularly relevant are the studies carried out to investigate the affect of mutant SOD1 expression in astrocytes (Julien 2007). Mutations in the super oxide dismutase 1 gene (*SOD1*) are causative for about 20% of all familial cases of Amyotrophic Lateral Sclerosis (ALS). Chimeric mice expressing mutant SOD1 in some but not all cells were found to exhibit motor neuron degeneration. Interestingly, a number of the affected and degenerating motor neurons were not themselves expressing mutant SOD1 (Clement *et al.* 2003). Cell culture is a good technique to further explore the link between SOD1 expression and neuronal degeneration. Different cell types either expressing mutant or wildtype (wt) SOD1 can be co-cultured and the results carefully monitered at all time points. By having better control of the conditions and the types of neuronal cells present, cell culture offers advantages that no *in vivo* system can. Nagai *et al* (2007) found that mutant SOD1 expressing neurons grown alongside astrocytes expressing mutant SOD1 were degenerating faster than those surrounded by wt astrocytes or even other types of cells whether or not they expressed mutant SOD1. They also found that conditioned media derived from mutant astroctyes could affect the survival of motor neurons which supported the idea of a soluble factor being released from the astrocytes (Nagai *et al.* 2007).

Cell culture may be a good way to dissect individual pathways but to model a disease and look at the way a mutated gene or protein acts in a system, with the associated stochastic changes and variables, *in vivo* is the only way. For a system that has many genetic tools available and a fully characterized genome, *Drosophila melanogaster* is ideal.

1.3 Modeling disease in *Drosophila melanogaster*

More than 50% of human genes are believed to have a *Drosophila* homologue with some entire pathways being conserved and as many as 75% of disease causing genes are believed to be conserved (Fortini *et al.* 2000; Rubin *et al.* 2000; Bonini and Fortini 2003). The similarity in phenotype between humans and flies expressing a disease mutation is evidence of the conservation of the underlying disease pathways. The short lifespan of *Drosophila* means that a new generation can be produced every two weeks and flies require a simple diet and few essential requirements so they can be kept comparatively cheaply. Mutants can be easily generated through chemical mutagenesis e.g. Ethyl methanesulphonate, or P-element insertional mutagenesis (the disruption of a gene by the insertion of a transposable element into the genome). For the ease and benefit of the majority of the *Drosophila* community, stocks of flies carrying carefully mapped gene mutations and transposon insertions are readily available to order from stock centres. Finally, it is easy to feed flies pharmacological agents by mixing them with the food as larvae are not fussy eaters.

During the last century, not only has *Drosophila* become an increasingly important tool for genetic studies, in recent years it has also become a very useful model for studying neurodegenerative diseases (St Johnston 2002; Driscoll and Gerstbrein 2003). A large number of the disease genes in humans have been evolutionary conserved and homologues for the majority of human genes can be found in *Drosophila* (Bonini 2001; Bonini and Fortini 2003). Through the mutation of an endogenous homologue or the transgenic expression of the human gene, models have already been created for many diseases including Polyglutamine Disease, Parkinson's Disease and Alzheimer's Disease (AD) (Bonini and Fortini 2003; Driscoll and Gerstbrein 2003; O'Kane 2003). Expression of genes in the fly can be controlled by the utilization of the yeast GAL4 activation system.

1.4 *Drosophila* and the GAL4 system

The yeast (*Saccharomyces cerevisiae)* GAL4 transcription factor binds to a specific Upstream Activating Sequence (UAS) to promote the transcription of genes under its control (Brand and Perrimon 1993). In 1993, Brand and Perrimon described the use of this method in *Drosophila* to spatially and temporally target the expression of a gene. The approach is bipartite, two separate parental lines express either the GAL4 in a particular spatial pattern (driver) or they have the gene of interest under control of a UAS (responder), see Figure 1.1. When the two constructs are separate, the gene is silent and the two stocks are phenotypically normal. When flies are crossed between the two lines the offspring will have the UAS-gene x and will produce GAL4 in a tissue and time of interest. This will bring about the expression of gene x but only in the pattern dictated by the expression of GAL4 (Brand and Perrimon 1993; Duffy 2002). Driver lines are available with a wide array of expression patterns, including ones expressing only in motor neurons, those that are pan neural and a number that express only in the eye.

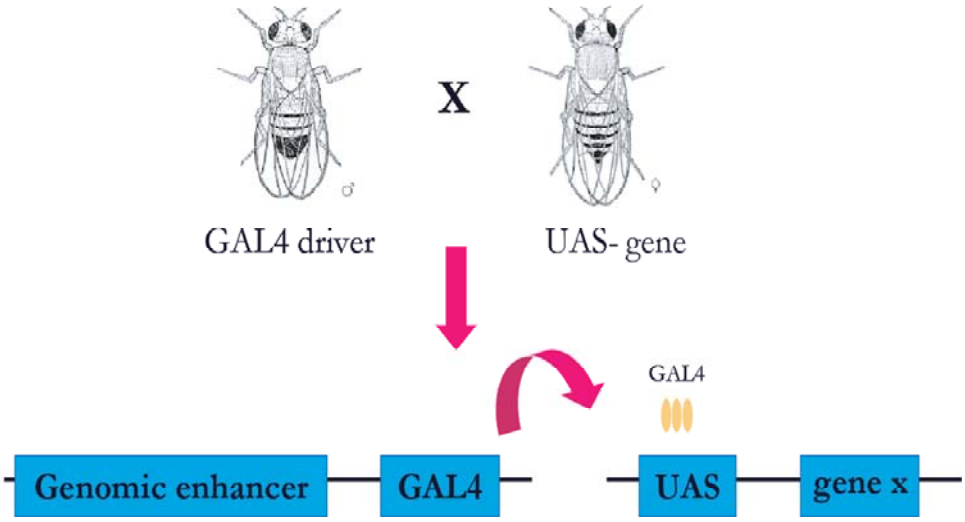


Figure 1.1: The GAL/UAS system.
A male carrying the GAL4 driver is crossed with a female carrying the UAS responder (UAS-gene x). The resulting progeny contain both elements and express gene x in the pattern dictated by the expression of GAL4

GAL4 is temperature sensitive and by simply altering the temperature in which the flies are raised, a variety of expression levels for the UAS-gene can be achieved. This can be particularly useful if low levels of protein expression are necessary to overcome an early developmental stage after which a higher expression level can be sustained. Flies are raised at 29^{o}C to ensure optimum GAL4 activity while maintaining fly viability. Since the discovery of the GAL4/UAS system, modifications to further optimize the transcription of a gene both temporally and spatially have been created. These tend to be tripartite approaches and one such method is the use of hormone responsive GAL4 chimeras. By putting GAL4 activity under the control of progesterone or estrogen, the ability of GAL4 to induce the expression of the responder is more accurately controlled at a temporal level by the exposure of the GAL4-UAS progeny to the hormone (Duffy 2002).

Examining genes linked to degeneration would prove far more difficult if it wasn't possible to limit the expression pattern within the fly as causing widespread degeneration may simply lead to premature death before any informative research can be carried out. It is possible to create mosaics in which only certain cells are affected and thereby the fly is able to survive whilst still providing insight into the deleterious effects of the gene in the affected cells. However, a simpler and more common option in the modeling of disease is to drive expression solely in the adult eye.

1.5 Modeling disease in the eye

The insect eye is a complex but uniform structure which has been well characterized allowing any modifications to be easily measured and recorded. More importantly it is not essential to the survival of the fly so degeneration of the eye can be followed over a period of time, important when studying age-dependent diseases. The compound eye is composed of roughly 800 ommatidia and degeneration of a number of these produces a rough eye phenotype of varying severity (Bonini and Fortini 2003). The beauty of the GAL4/UAS system in flies allows the spatial expression of genes to be restricted to just

the eye and as such the degenerative phenotype of any gene can be looked at solely in this organ. In addition to this, modifier screens can be carried out to examine genes that affect a rough eye phenotype in an enhancing or suppressing fashion.

Spinocerebellar Ataxia 8 (SCA8) is a triplet expansion-induced neurodegeneration which is unique in that the expansion occurs within an entirely non-coding transcript. A model of the disease was produced by expressing the human SCA8 non-coding RNA in the fly eye under the control of the glass multiple reporter (*gmr*)-*GAL4* driver and the result was a rough eye phenotype with disorganized ommatidia and mechano-sensory bristles of the eye (Mutsuddi *et al.* 2004). Once this eye phenotype was characterized the next step was to look for enhancers or suppressors of the phenotype and this was done by crossing these lines with fly stocks containing characterized P-element insertions known to disrupt particular genes. This technique is called an enhancer-suppressor screen and the idea is to use a particular phenotype, such as a rough eye, to screen for genetic interactors that influence this phenotype. Suppression is seen when there is a decrease in the severity of the rough eye or a complete reversion to wild type. An enhancement is an increased severity, greater roughness and often a decrease in eye size. Such a suppression or enhancement suggests that the disruption of a particular gene is somehow affecting the phenotype brought about by the mutant gene x, or in this case non-coding RNA. It indicates that there is a genetic interaction between the two genes; either they act in the same pathway or in a parallel process. It does not mean that they directly interact physically although that may be the case. The rough eye is an ideal phenotype to easily screen thousands of flies making it possible to check for a large number of genetic interactors very quickly. To increase the speed of a screen, there are P-element insertion lines that disrupt one or only a few genes at once so a number of genes can be screened for a possible interaction and then, if there is a change in the phenotype, the specific gene can be narrowed down by obtaining smaller insertions for the chromosomal region. Such a screen is a good compliment to biochemical approaches looking at direct protein interactions.

Various other triplet repeat expansion induced neurodegenerative diseases have been modeled in *Drosophila* and again the eye has proven to be an ideal organ in which

to focus the expression of the disease protein. In an effort to model HD, Jackson *et al* (1998) expressed fragments of human huntingtin in the *Drosophila* photoreceptor cells under the control of the *gmr-GAL4* driver. Similarly, Bonini (1999) modeled the human disease Spinocerebellar ataxia 3 (Machado-Joseph disease) by expressing the human protein containing the disease causing triplet repeat both in the fly eye (*gmr-GAL4*) and pan neurally (*elav-GAL4*). In Bonini and Jackson's eye models, the flies containing the mutant form of the protein showed a number of major hallmarks of the disease, most notably a late onset degeneration of the photoreceptor neurons and a nuclear accumulation of the mutant protein (Jackson *et al.* 1998; Bonini 1999). This is in contrast to controls expressing wt human protein in which the triplet repeat is within a normal range as these flies have no noticeable defects. Despite this correlation there are a few differences between the models and this is attributed to a difference in approach to the spatial selection of gene expression. Bonini used the GAL4/UAS system as previously outlined so the expression of the gene is reliant on the production of GAL4 which in turn is driven by the *gmr* promoter (Bonini 1999). Jackson *et al.* had huntingtin under the direct control of the *gmr* promoter which bypassed the need for GAL4 and as such might have seen an earlier production of the protein in the fly (Jackson *et al.* 1998). This is an example of the flexibility of *Drosophila* as a model system. A number of different methods are available to bring about a similar goal and the choice taken will depend on the type of gene, its function and perhaps simply a time and ease factor. Very slight changes can be made to the expression of genes and a number of modifications can be brought together in a single fly fairly easily and comparatively quickly. The benefit of *Drosophila* has been realized by the community of scientists working on degenerative disease as is evident by the number of diseases modeled in the fly (Bonini and Fortini 2003).

1.6 *Drosophila* models of neurodegenerative disease

Disease	Gene	Method	Phenotype	Author
Alzheimers Disease	Aβ 42	Expression of human Aβ 42 in *Drosophila* eye and pan neurally	Photoreceptor degeneration, locomotion defects and aggregation.	(Finelli *et al.* 2004; Iijima *et al.* 2004; Iijima and Iijima-Ando 2008))
Tauopathy	Human Tau	Expression of mut human tau	Shortened lifespan, degeneration in the adult brain	(Wittmann *et al.* 2001)
ALS	SOD1	Expression of human mutant SOD1	Locomotion defects	(Watson *et al.* 2008)
ALS	SOD1	Null cSOD mutant	Reduced lifespan, reduced fertility and increased sensitivity to Paraquat	(Phillips *et al.* 1989)
ALS	VAPB	Expression of mutant DVAPP56S	Aggregate formation, degeneration, locomotion defects	(Chai *et al.* 2008)
ALS	VAPB	Expression of mutant DVAPP56S	Aggregation, Disruption of synaptic homeostatis and signaling	(Ratnaparkhi *et al.* 2008)
ALS	VAPB	Expression of mutant DVAPP56S	Aggregate formation, ER disruption	(Tsuda *et al.* 2008)
ALS	TDP43	Knock out endogenous gene	Eclosure and locomotion defects	(Feiguin *et al.* 2009)
ALS	TDP43	Expression of human TDP43 in adult eye and larvae motor neurons	Disruption of eye rhabdomeres and locomotion defects in larvae leading to death	(Hanson *et al.*; Li *et al.*; Lu *et al.* 2009)
ALS	TDP43	Expression of human TDP43 in adult eye and larvae CNS	Disruption of Rhadomeres, axonal loss and cell death	(Li *et al.*)
ALS	TDP43	RNAi of TDP43 and expression of mutant human TDP43	Reduction in dendritic branching	(Lu *et al.* 2009)
Parkinson's Disease	α synuclein	Pan neural expression of human α synuclein	Locomotion defects and dopaminergic neuronal degeneration	(Feany and Bender 2000)

Parkinson's Disease	α synuclein	Pan neural expression of human α synuclein	Dopaminergic neuronal degeneration	(Trinh *et al.* 2008)
Parkinson's Disease	Parkin	Knock out of endogenous protein	Reduced longevity, locomotor dysfunction, muscle and neuronal degeneration	(Greene *et al.* 2003)
Parkinson's disease	Parkin	Expression of mutant human Parkin	Locomotor defects and neuronal degeneration	(Yang *et al.* 2003; Pesah *et al.* 2004; Whitworth *et al.* 2005; Sang *et al.* 2007)
Parkinson's disease	Pink1	Knock out of endogenous protein	Mitochondrial dysfunction and dopaminergic neuronal defects	(Clark *et al.* 2006; Park *et al.* 2006)
Parkinson's disease	Pink1	dPink1 RNAi	Dopaminergic neuronal loss muscle degeneration, mitochondrial defects and ommatidia degeneration	(Wang *et al.* 2006; Yang *et al.* 2006)
Parkinson's disease	DJ1	Knock out of endogenous proteins α and β	Increased sensitivity to oxidative stress.	(Menzies *et al.* 2005; Meulener *et al.* 2005; Yang *et al.* 2005)
Parkinson's disease	DJ1	RNAi knock down of DJ-1 α	Loss of photreceptors, dopaminergic neuronal disruption and increased sensitivity to oxidative stress	(Yang *et al.* 2005)
Parkinson's disease	LRRK2	Expression of mutant *Drosophila* LRRK	Dopaminergic degeneration and increased sensitivity to oxidative stress	(Imai *et al.* 2008)
Parkinson's disease	LRRK2	Transgenic expression of human mutant LRRK2	Locomotion defects and dopaminergic neurodegeneration	(Ng *et al.* 2009)
Parkinson's disease	LRRK2	Transgenic expression of human mutant LRRK2	Retinal degeneration, locomotion defects and loss of dopaminergic neurons	(Liu *et al.* 2008b)
PolyQ disease	SCA3/ MJD trQ78	Transgenic overexpression in adult eye and pan neurally	Degeneration and NI formation. Behavioural defects.	(Warrick *et al.* 1998; Bonini 1999; Kim *et al.* 2004)

PolyQ disease	SCA3/ MJD trQ78	Transgenic expression in glia	Motor impairments and degeneration	(Kretzschmar *et al.* 2005)
PolyQ disease	SCA8	Transgenic expression in adult eye	Photoreceptor degeneration	(Mutsuddi *et al.* 2004)
PolyQ	Huntingtin	Expression of polyQ expanded Huntingtin in adult eye	Photoreceptor cell degeneration	(Jackson *et al.* 1998)
PolyQ	Huntingtin	Glial targeted expression of himan Huntingtin	Locomotor defects and neuronal dysruption	(Tamura *et al.* 2009)

Table 1.1 Summary of *Drosophila* models of human neurodegenerative disease

The second most common human neurodegenerative disease following AD is PD (Auluck *et al.* 2002) Like the polyglutamine diseases, PD has also been modeled in *Drosophila* and a number of different techniques have been used. The original model used the UAS/GAL4 system to over express the human α-synuclein gene in *Drosophila* (Feany and Bender 2000). Mutations in α-synuclein were first linked to cases of familial PD in a large Italian family (Polymeropoulos *et al.* 1997). In the same year it was also shown to be a major component of the intraneuronal inclusions known as Lewy bodies that are a major hallmark of familial and sporadic PD (Spillantini *et al.* 1997). The function of α-synuclein has not been fully described but it is located in the nucleus and at the synapse where it is believed to have a role in synaptic plasticity and neurotransmission (Abeliovich *et al.* 2000; Bonini and Giasson 2005).

Recently Chandra *et al* showed that α-synuclein has the ability to rescue the lethality associated with the knockout of Cysteine String Protein alpha (CSPα) (Chandra *et al.* 2005). CSPα is a molecular chaperone necessary for the integrity of synaptic nerve terminals. CSPα-deficient mice have a significant reduction in synaptic SNARE protein complexes and present with degeneration of the axon terminal at the neuromuscular junction (NMJ) which leads to death by 3 months of age. Transgenic expression of α-synuclein rescued the degenerative phenotype so that mice lived longer than 15 months whereas removal of endogenous α-synuclein accelerated the lethality

associated with removal of CSPα (Chandra *et al.* 2005). This indicates that α-synuclein acts downstream of CSPα and has a protective effect against degeneration. This is an important finding as the toxic form of α-synuclein that is causative for some familial cases of PD is thought to be a gain of function mutation as it is an over expression of α-synuclein that leads to degeneration in the models of PD. Deletion of α-synuclein has little morphological or behavioral effect on otherwise wt mice, without other mutations as described by Chandra *et al* (Gureviciene *et al.* 2007).

The mutant forms of α-synuclein that have been identified as causative for PD are known as A30P and A53T which relates to the position of the amino acid affected. To model the disease both of these mutant human proteins were individually expressed in the fly. A third line was created expressing the wt human protein (Feany and Bender 2000). Interestingly in all three cases, there was an age dependent loss of dopaminergic neurons when the protein was expressed using a pan neural driver. A punctuate staining pattern for α-synuclein also showed that aggregates were forming that were similar in morphology to the Lewy bodies seen in human patients. These inclusions were present only in the nervous system but were in various subsets of neurons and not restricted to the dopaminergic cells despite these being the only ones showing degeneration. This is similar to the situation we see in human patients (Feany and Bender 2000). This being considered, it is also interesting that expression of α-synuclein in the eye driven by *gmr-GAL4* also caused a degenerative phenotype restricted to the eye. As this is a stronger driver it may be that the eye is not as susceptible to α-synuclein and an increased amount of the protein is necessary to induce degeneration as compared to the vulnerable dopaminergic neurons. This is consistent with the fact that wild type and mutant proteins gave a comparable phenotype. It is perhaps the accumulation of the protein due to the over expression that drives degeneration. In humans the wild type protein is also present and the mutant protein may resist degradation and thereby accumulate and form the aggregates that are a hallmark of this and so many other degenerative diseases.

Further investigation into PD using mice and *Drosophila* supports the hypothesis that protein accumulation is the key to this disease. In mice, the overexpression of wt α-synuclein induces the formation of ubiquitin positive inclusions (Masliah *et al.* 2000).

In *Drosophila,* the co-expression of Parkin, a ubiquitin ligase that targets proteins for degradation, in particular α-synuclein, rescues the phenotypes associated with α-synuclein expression. Both the motor dysfunction and the eye phenotypes are suppressed by an overexpession of the *Drosophila* Parkin (Haywood and Staveley 2004) and similarly with the expression of PTEN-induced putative kinase 1 (Pink1) which is thought to act upstream of Parkin (Todd and Staveley 2008). Pink1 is also known to activate chaperones and this may be a further pathway through which it acts because the chaperone HSP70 has been shown to suppress many neurodegenerative diseases including Parkinson's Disease (Auluck *et al.* 2002). Not only are Parkin and Pink1 protective but mutations in these proteins are causative for autosomal recessive juvenile cases of Parkinson's Disease. These cases have an onset between the ages of 20 and 40 years but otherwise they are identical to sporadic cases (Kitada *et al.* 1998; Valente *et al.* 2004; Albanese *et al.* 2005; Gasser 2009).

A model of Parkinson's Disease has been created by knocking out the endogenous homologue for Parkin in *Drosophila* and, in an alternative model, expressing human mutant Parkin in a wt background (Greene *et al.* 2003; Cha *et al.* 2005; Whitworth *et al.* 2005; Wang *et al.* 2007). Inactivating the endogenous gene to model a disease as in the case of Parkin demonstrates further the flexibility of *Drosophila* as a model system. Inactivation of Parkin lead to locomotion defects due to apoptotic muscle degeneration which was thought to be due to defects in mitochondria as seen by the swelling of these organelles and a disintegration of the cristae. Such degeneration of the mitochondrion was seen in flight muscles prior to any other morphological changes which suggests this is an early indicator of disease (Greene *et al.* 2003). Interestingly there was select degeneration in the dorsomedial dopaminergic cell cluster which is also an area of enhanced toxicity in the *Drosophila* α-synuclein model (Auluck *et al.* 2002).

In an attempt to elucidate the mechanisms of the observed phenotype in the Parkin mutant, Greene *et al.* went on to screen for the potential pathways involved by microarray and looking for alterations in the level of transcription of about 6000 genes. Genes that were significantly up/down regulated were verified by crossing mutants in

which the specific gene is knocked out with a homozygous *Parkin* null fly that shows partial lethality. Enhancement or reduction in the lethality was used to confirm genetic interactions and following the success of this screen Greene *et al.* went on to screen for other genetic interactors using a collection of enhancer P (EP) transposon insertion lines. (Greene *et al.* 2005). When P elements are inserted into the *Drosophila* genome they tend to be inserted at the 5' end of a gene and often upstream of the open reading frame. As such although in some cases the gene is disrupted resulting in a loss of function, in others the transcription of the gene is possible and a fully functional protein can be produced. The EP lines use a transposon containing a GAL4 responsive promoter and the insertion can actually drive the overexpression of the downstream gene when crossed with a driver line. Once a modifier of the *Parkin* lethality phenotype was observed the insertion was then examined to see if it was due to an over expression or loss of function. Two loss of function enhancers that were recovered from this screen were *Glutathione transferase 1* and *thioredoxin*, both involved in oxidative stress (Greene *et al.* 2005). This shows how *Drosophila* can be used to model a disease by either expressing a human mutant disease protein through the incorporation of the gene into the fly's own genome or by removing the endogenous protein. In the case of Parkin, Greene *et al.* looked at the fly's endogenous protein and the genetic interactions it formed *in vivo* and now this can be transferred to the human cases to see if similar pathways are also affected. Consistent with these data, patients with Parkinson's disease have been shown to have reduced levels of glutathione (Sian *et al.* 1994).

Not only is *Drosophila* a good model for disease but with all the tools available it is easy to screen for modifiers that can affect the disease onset and progression and because of the high level of conservation between species, many of the outcomes can be directly translated to human patients. Due to the complex hereditary and variability in humans, finding the causative gene for a disease is a much more difficult task. A disease that has been a cause of much research is Amyotrophic Lateral Sclerosis (ALS). The majority of cases are sporadic and as such it has been difficult to model ALS. However with the discovery of familial cases linked to mutations in SOD1, the

possibility arose to model the disease and led the way to finding other contributing genes.

1.7 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is the most common type of motor neuron disease, characterized by progressive degeneration of the upper and lower motor neurons. Sufferers present with symptoms of muscle weakness and this quickly develops on to paralysis and finally death due to respiratory failure within 5 years of disease onset (Hadano *et al.* 2001; Farah *et al.* 2003). The incidence of the disease is 1-2 per 100,000 with a lifetime risk of 1 in 2000 and apart from a few exceptions these figures are fairly uniform all over the world. The Chamarro population of Guam is one such exception that has a higher than average incidence of ALS termed Guam syndrome (Ince and Codd 2005). This familial form of disease is linked to a dementia similar to Parkinsonism. The cause of this and a few other small areas of high incidence disease populations are unknown but there are a number of environmental and genetic hypotheses. The most popular but also largely contested theory is that the disease is a result of toxicity from β-methylaminoalanine (BMAA) a product of the symbiotic cyanobacteria which lives in Cycad trees (Karamyan and Speth 2008). The seeds of this tree contain the toxin BMAA and are used by the Chamarro population in food and medicine. It is argued that levels ingested are very low but through the consumption of the fruit bat which lives on the seeds it is possible that there is a build up of this toxin to a dangerous level. The disease presumably has a strong genetic basis also, whether this is a susceptibility to the toxin or a direct cause of the disease (Sieh *et al.* 2009). In the rest of the world the majority of cases are sporadic but similar to Guam about 10% are familial (Nishimura *et al.* 2004a). The most common of these familial cases are those caused by a mutation in the *Superoxide dismutase* (*SOD1*) gene.

1.8 Familial ALS

1.8.1 SOD1

SOD1 is a gene encoding the enzyme Superoxide Dismutase that catalyses the conversion of the highly reactive oxygen free radical (O_2^-) into hydrogen peroxide and O_2 and it is mutations in this gene that have been linked to 20% of all familial cases of ALS (Rosen 1993; Takamiya *et al.* 2005). There are over one hundred known mutations in *SOD1* that lead to ALS and the majority are missense mutations producing a shorter protein product. The mutant alleles are dominant and cause the disease in heterozygosity despite there being a fully functioning wt protein. This, in conjunction with the evidence from mouse models in which the human mutant transgene causes MND despite their own SOD1 being intact, indicates that the mutation is not a loss of function. It is believed that SOD1 takes on a new toxic function possibly quite separate from its normal role and thus, despite it being a well characterized protein, there is still little known about the pathogenesis of SOD1-linked ALS (Farah *et al.* 2003; Takamiya *et al.* 2005). In order to dissect the molecular basis of this disease a number of models have been created using transgenic mice and rats expressing mutant *SOD1*. Gurney *et al* were the first to undertake the challenge of creating an SOD1 mouse by expressing the mutant human G93A allele in mice. The resulting progeny showed an age-dependent paralysis in one or more limbs, tremor in the hind limbs and when examined at a morphological level showed a decrease in staining for Choline Acetyltransferase indicative of neuronal loss. Gurney *et al* also found that muscle endplates were multiply innervated and single axons formed numerous contacts suggesting that there is a sprouting mechanism to compensate for the early degeneration (Gurney *et al.* 1994).

A number of models have since been created and used to study all aspects of disease onset and progression. They have proved invaluable tools for increasing our understanding of the disease, especially in studying the early pre-symptomatic stages, something which, for obvious reasons, is difficult to do in humans. The retardation of

slow axonal transport has been reported to occur in SOD1 mutant mice months before signs of degeneration and reduced levels of microtubule associated proteins, MAP-1 and MAP-2, and an altered level of tau phosphorylation has also been described well before the onset of symptoms (Williamson and Cleveland 1999; Farah *et al.* 2003). The mutant SOD1 protein can associate with actin and is found in the aggregates that form in the motor neurons of transgenic mice (Takamiya *et al.* 2005). Cytoplasmis inclusions are a major hallmark of disease and SOD1 is the main component of these aggregates in human patients suffering from type 1 familial ALS. A closer look at the formation of these aggregate has shown there to be two different types; detergent soluble and larger in-soluble forms. In transgenic mice, these larger insoluble forms of mutant SOD1 are more prevalent in vulnerable tissue and only when the disease is rapidly progressing. This may mean that they play a causative role in the disease but as a number of pathologic features are already present prior to their observation it is likely that they are more involved in disease progression than onset (Karch *et al.* 2009). It is possible that very low levels of these insoluble aggregates are necessary to initiate disease or it may be that it is the smaller forms that promote the disease and in due course, large aggregates form as the cell starts to malfunction. The appearance of aggregates has been implicated as an indication of chaperone and proteasome system malfunction. Under normal circumstances, the cell is protected from accumulated proteins by chaperones that either aid the normal refolding or target the abnormal proteins to the proteasome for degradation (Kabashi and Durham 2006). As it has been found that proteasome inhibitors can induce the formation of SOD1 insoluble inclusions when added to cell culture, it is certainly possible that the proteasome system is not working properly in ALS patients (Kabashi and Durham 2006).

A recent and important finding by Clement *et al.* (2003) was that of the protective effect exerted by wild type neurons in chimeric mice expressing mutant human SOD1 in a subset of neurons. The number of mutant SOD1 expressing cells degenerating was inversely proportional to the number of wt neurons in the region and chimeric mice showed a delayed disease onset and a longer lifespan as compared to $SOD1^{G93A}$ littermates (Clement *et al.* 2003). Perhaps more interesting is the fact that

wild type neurons of older chimeras developed ubiquitin-positive aggregates suggesting the disease eventually spreads from mutant neurons to healthy ones. Whether there is a soluble factor that could promote degeneration was later confirmed in cell culture by Nagai *et al* (2007) as described previously.

Despite some interesting research coming from mouse and rat models, the fly models have not been so successful. The most promising is from Watson *et al.* (2008) in which the mutant flies expressed a mutant human SOD1 protein and showed signs of motor deficits, synaptic transmission reduction and upregulation of the chaperone heat shock protein 70 (Watson *et al.* 2008). The expression of the wt protein also gave these phenotypes but, as in many disease models discussed earlier, it may be the level of protein that is important and it is the overexpression of SOD1 causing the toxic affect on the cell. In this case it may also be due to the difference between the *Drosophila* SOD1 and the human protein as there is a variance of 49/153 residues at the amino acid level (Watson *et al.* 2008). Such a variance from the endogenous protein might cause the fly system to treat the human protein as toxic in a similar manner to the mutant protein and as such it would be preferable to express a mutated copy of the *Drosophila* protein. Unfortunately this does not recapitulate any of the disease hallmarks. Lack of any visible neuronal loss is also concerning but but there was electrophysiological data which suggested synaptic dysfunction. It may be that the primary pathological event occurs at the synapse meanwhile the cell body remains comparitively intact.

1.8.2 ALS2

Another familial linked form of ALS is due to mutations in *ALS2*. *ALS2* encodes Alsin, a 184 kDa protein with three guanine nucleotide exchange factor (GEF) domains and mutations in this gene lead to a recessive juvenile form of ALS termed ALS2 (Hadano *et al.* 2001; Hadano *et al.* 2006). The protein contains regions that are highly homologous to RCC1, a GEF for the GTPase Ran that has roles in nuclear transfer and microtubule assembly (Yang *et al.* 2001; Hadano *et al.* 2006). Alsin also contains a tandem

organization of diffuse B-cell lymphoma (Dbl) homology and pleckstrin homology (PH) domains. This is a feature of GEFs for Rho GTPases implicated in signaling cascades, neuronal morphogenesis, membrane transport and actin cytoskeleton (Hadano *et al.* 2001). It is hypothesized that mutations disrupt the modulatory function in Ran/Rho-related GTPases and this in turn causes defects in microtubule assembly, membrane organization and trafficking. Since its discovery, 6 independent groups have created *ALS2* -/- knockout mice. Results from these models have been rather mixed, with varying degrees of motor deficits possibly accountable to the differing recording methods and mice with varying genetic backgrounds.

The first model created by Hadano *et al.* (2006) developed subclinical deficits of spinal motor neurons and showed a significant decrease in cerebellum Purkinje cells but it demonstrated normal motor performance, growth and reproductivity (Hadano *et al.* 2006). In all ALS2 models, any visible motor defects are only moderate and lower motor neurons are never affected by the disease. This correlates with the human disease as the majority of cases predominantly affect the upper motor neurons but the difference in neural organization might mean that mice motor capabilities are less affected. The rodent upper motor neurons always interact with an interneuron in the spinal cord whereas human neurons can directly innervate the lower motor neurons to coordinate movement. This being said, in the lateral column, a progressive degeneration of the corticospinal tract (CST) neurons was evident in a model created by Yamanaka *et al* (2006). However, the age-dependent slowly progressive loss of Purkinje cells in the cerebellum of the knock out mouse created by Hadana *et al* was not evident in Yamanaka's model. (Hadano *et al.* 2006; Yamanaka *et al.* 2006) Cai *et al* created another model and also found no evidence of Purkinje cell loss but they did note a decrease in cerebellum-mediated motor learning when the mice were at 20 months of age (Cai *et al.* 2005).

Despite the discrepancy between some of the results from the different models there is some promising data and in particular they are shedding light on the normal function of the protein. As it is a recessive allele the mutation is almost certainly due to a loss of function so knowing the role of this protein is vitally important. Despite Cai *et*

al finding no neuropathological abnormalities they did find a role for Alsin in neuroprotection from toxic challenges. The toxin PQ herbicide produces reactive oxygen species which are very harmful to cells and following exposure at a level which left wt mice unharmed, 50% of *ALS2*-/- mice died. Post mortem showed edema and hemorrhage in the lungs. This led Cai *et al* to conclude that *ALS2* mutations confer a susceptibility to oxidative stress and should be classed as a risk factor (Cai *et al.* 2005).

1.8.3 ALS 4

ALS4 is another rare familial juvenile onset form of ALS which presents with limb weakness, severe muscle wasting and pyramidal signs. There is however an absence of overt sensory abnormalities and bulbar and respiratory muscles are not affected. Sufferers develop symptoms in childhood but often have a normal lifespan. In 2004 a large Maryland family enabled Ying-Zhang *et al* to map the gene responsible to *Senataxin (SETX,)* a gene encoding a novel DNA/RNA helicase. These are a family of proteins known to be involved in DNA repair, replication, recombination and transcription and RNA transcript processing (Chen *et al.* 2004). The linkage to this gene was verified by a number of other families and the responsible mutations were found to be missense and lead to a single amino acid change in the protein product. Unfortunately no solid understanding of the function of this protein is known but interestingly it has been linked to another degenerative disorder called Ataxia-ocular apraxia 2 which presents with cerebellar atrophy, sensory-motor neuropathy and oculomotor apraxia (Moreira *et al.* 2004). Any increase in our understanding of the role of *SETX* in either of these disorders will be beneficial to both parties and the different diseases will provide a greater number of affected individuals available to study these rare cases.

1.8.4 ALS 11

In 2009 dominant mutations in the *FIG4* gene were found be to linked to a number of cases of ALS termed ALS11 (Chow *et al.* 2009). FIG4 is also known as SAC3 and is a phosphoinositide-5-phosphatase. This is one of a family of SAC domain phosphatases that catalyses the removal of a phosphate group from the lipid signaling molecules phosphoinositide phosphates. SAC domain proteins have a region of about 400 amino acids with a high level of homology to the yeast Sac1p (Hughes *et al.* 2000). The gene *SAC1* (Suppressor of actin) was so-called because yeast mutants suppressed the actin phenotype associated with the temperature-sensitive actin mutation act1-1^{ts} (Novick *et al.* 1989). There are seven highly conserved motifs and in particular a stretch of amino acids in the 6th domain that is thought to be the catalytic phosphatase domain.(Hughes *et al.* 2000) The reversible phosphorylation of phosphatidylinositol at the 3rd, 4th or 5th head group position generates different phosphoinositide species (Volpicelli-Daley and De Camilli 2007) (See figure 1.2). A total of 7 Phosphatidylinositol phosphates (PIPs) can be created with various combinations of phosphorylation and they are each recognized as a different species each with a different cellular localisation and function (Volpicelli-Daley and De Camilli 2007). They have roles in the recruitment of other signaling molecules to the cytosolic membrane surface and as such the phosphatases and kinases that regulate the levels of the different proteins play an important role in ensuring correct levels are maintained. FIG4 is a lipid phosphatase that removes the phosphate group from the 5th head group of PI(3,5)P_2 to create PI(3)P. (Volpicelli-Daley and De Camilli 2007; Botelho 2009)

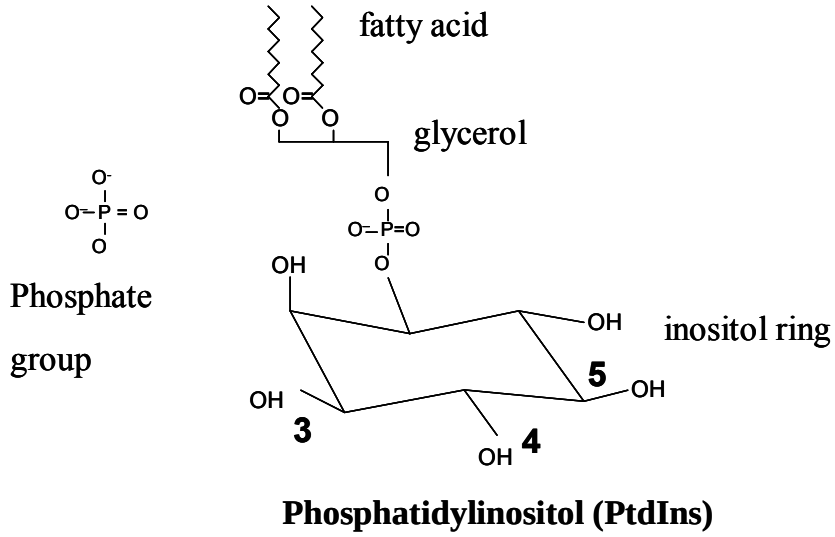


Figure 1.2 Basic structure of the Phosphatidylinositol Phosphate (PIP)
PIPs are created by the addition of a phosphate group onto the inositol ring of phosphatidylinositol (Ptdlns) at position 3,4 and/or 5. Phosphates are added by a PI kinase and removed by a PI phosphatase. (Diagram recreated from Hughes *et al* 2000).

Mutations of the human *FIG4* gene have previously been reported to be responsible for a recessive severe form of Charcot-Marie-Tooth disease (CMT), a peripheral neuropathy that affects both sensory and motor neurons. (Chow *et al.* 2009) The motor neuron dysfunction seen in the CMT patients prompted a screen for cases of ALS caused by *FIG4* in a candidate gene approach. In a screen of almost 500 ALS patients and more than 400 control individuals, 10 variants of the *FIG4* gene were found in the patient group that were not present in the control. Of these, 6 are recognized as deleterious due to the incorporation of a premature stop codon before the catalytic domain or a deletion within this domain. All the patients were heterozygous and have a wild-type allele as well as the mutated version (Chow *et al.* 2009). This might be the reason for the difference in disease phenotype of these ALS patients as compared to the CMT patients who are homozygous and develop the disease in childhood or early adulthood.

In mice there is a pale tremor phenotype that is caused by mutations in the *FIG4* gene and interestingly studies of the $PI(3,5)P_2$ levels in these mice show that rather than being elevated they are actually at a lower level (Chow *et al.* 2007). This is also true for

yeast that are deficient in FIG4 and the possible explanation is that it is due to the disruption of the interaction with the FAB1 activator VAC14. In yeast FAB1 is the kinase responsible for the synthesis of $PI(3,5)P_2$ and it is activated by VAC7 and VAC14. An interaction between VAC14 and FIG4 has been shown and the possibility that this plays a role in the maintenance of the $PI(3,5)P_2$ level by controlling the ability of VAC14 to activate FAB1 is still being confirmed. In addition to this there are a number of PI-5-phosphates that can remove the 5^{th} phosphate group and replace the need for FIG4 to produce $PI(3,5)P_2$ (Dove *et al.* 2002; Gary *et al.* 2002) Interestingly mutations in VAC14 also cause a neurodegenerative phenotype with neurological defects in mice. (Zhang *et al.* 2007).

1.8.5 TDP-43

In 2006 a major component of ubiquitinated aggregates found in many patients with sporadic ALS was discovered to be a 43kDa protein called Transactive Response (TAR) -DNA binding protein (TDP-43).(Neumann *et al.* 2006; Kwong *et al.* 2007) These aggregates, known as ubiquitinated inclusions (UBIs) are found in the cytoplasm and nucleus of neural and glial cells of sporadic cases of ALS and a number of familial. They are also found in patients with the most common form of fronto-temporal dementia (FTLD-U) which is perhaps expected considering ALS is often associated with FTD. The mystery of the components of the UBIs was solved by Neumann *et al* who generated mouse antibody probes against insoluble brain tissue material from FTLD-U patients and then used liquid chromatography-tandem mass spectrometry to identify the positive residues (Neumann *et al.* 2006). To date the only exception is the inclusions in neurons of familial SOD1 patients as these are negative for TDP-43. Interestingly the characteristic inclusions of a wide array of other neurodegenerative diseases including Parkinson's and Trinucleotide repeat diseases are also negative for TDP-43 except for 30% of Alzheimer's disease (AD) patients (Kadokura *et al.* 2009).

TDP-43 is widely expressed in a number of tissues including lungs, heart and brain and it is normally found in the nucleus although the formation of aggregates in the cytoplasm of disease patients leads to a reduction of the nuclear localization. It is 414 amino acids in length and has two RNA recognition motifs (RRM1 and 2) and a glycine-rich C-terminal sequence. The original function designated to TDP-43 was as a repressor of *Tar-DNA* transcription which it achieves by binding to the polypyrimidine motif but recently it has also been found to affect transcription of other genes including *ApoA-II* and the mouse *SP-10* gene (Kwong *et al.* 2007). Following the discovery of TDP-43 in the inclusions many groups took up the task of finding dominant mutations in this gene that might be causative for familial cases of ALS. There are 6 exons within the TDP-43 open reading frame and the majority of disease mutations are dominant missense mutations in the sixth exon affecting the C-terminal sequence that is thought to be necessary for the interaction with other proteins (Kwong *et al.* 2007; Lagier-Tourenne and Cleveland 2009). The sequestering of TDP-43 away from the nucleus and into the cytoplasm may inhibit its ability to function and as such the mutation might be considered as a loss of function.

1.8.6 FUS/TLS – A new causative gene for ALS?

Following the discovery of the link between TDP-43 and ALS two groups independently identified mutations in another RNA/DNA binding protein that are also linked to familial cases of ALS (Kwiatkowski *et al.* 2009; Vance *et al.* 2009). Kwiatkowski *et al* carried out loss of heterozygosity (LOH) mapping on a family in Cape Verdean who had four members with ALS. The maternal Grandparents were first cousins and the population of the small island which they inhabited was only 6000 suggesting a recessive pattern of inheritance. A region on chromosome 16 was identified as having homozygosity, a region previously identified by Vance *et al* as linked to cases of ALS in a large British family that show a dominant pattern of inheritance (Vance *et al.* 2009).

The two groups narrowed down the culprit gene to *FUS/TLS*, (fused in sarcoma/translocated in liposarcoma). The dominant mutation R521C was also found in four other families and a number of other *FUS/TLS* mutations have been found in ALS patients making it likely that this is a new causative gene for ALS. *FUS/TLS* is 526 amino acids in length and is composed of 15 exons with an N-terminal domain rich in Glutamine, Glycine, Serine and Tyrosine, a glycine rich region, an RNA recognition motif, multiple RGG repeats implicated in RNA binding, a C-terminal zinc finger motif and a highly conserved extreme C-terminal region. The majority of mutations are missense and located in this terminal region (Lagier-Tourenne and Cleveland 2009).

Similar to TDP-43, the wt protein is localized in the nucleus and is expressed almost ubiquitiously. The disease protein although still found in nuclei is also present in cytoplasmic aggregates which is again similar to mutant TDP-43 but interestingly no TDP-43 positive aggregates are seen in the FUS cases suggesting that if the mechanism of disease pathology is similar, the pathways appear to be independent. (Vance *et al.* 2009) Investigation of FUS/TLS in mice found that it shuttles from the nucleus to dendritic spines and in agreement with this, knock out mice showed abnormal spine morphology. They hypothesized a role for this RNA/binding protein in synaptic plasticity through the regulation of RNA splicing and the transport of mRNA to local translationary machinery in the dendritic spine (Fujii *et al.* 2005).

1.9 VAPB and ALS

Recently a link was discovered between a missense mutation in the human *VAPB* gene (VAMP-associated protein-B) and some cases of ALS and Spinal Muscular Atrophy (SMA) (Nishimura *et al.* 2004a; Nishimura *et al.* 2004b). The mutation was a change of a proline to a serine at position 56 (P56S) and it was discovered in a large Brazilian family with 28 affected members. The same mutation was also found in another 6 related families and historical evidence historical evidence pointed to a single common ancestor. With over 200 affected individuals the evidence is strong for the mutant *VAPB*

as the causative agent. The mutation is autosomal dominant with VAPB localized on Chromosome 20 at position 20q13.3. In the original Brazilian family all affected family members have the same mutation but there is a wide variance in age of onset and the neurons involved so the disease manifested in the 28 individuals has been divided into three categories. See Table 1 below.

First, 8 patients are categorized as having late onset SMA. The age of onset is, on average, 46 years and the progression of disease is such that some individuals had suffered with the disease 10-12 years by the time the report was carried out. The disease is characterized by selective degeneration of the anterior horn cells in the spinal cord leading to muscle paralysis and atrophy but the lack of bulbar or pyramidal involvement separates this disease from ALS. Secondly, 15 affected family members presented with symptoms of atypical ALS. The average age at onset was 38 years and some of the affected members had suffered with the disease for 20-30 years indicating a slower progression as compared to the typical cases of ALS. These patients had the bulbar and pyramidal involvement of ALS but also a tremor that is atypical. Finally, 5 members had typical ALS and they presented with a late age at onset and a quick disease progression with death within 5 years.

Classification	No. affected	Neurons affected	Average age of onset	Disease progression
Late onset spinomuscular atrophy	8	Lower motor neurons	46	>10-12 years
Atypical ALS	15	Upper and lower motor neurons,	38	>10-20 years
Typical ALS	5	Upper and lower motor neurons	Late onset (exact age not disclosed)	< 5 years

Table 1.2 Summary of the ALS8 affected individuals
25 family members carried the VAPB P56S mutation and all manifested with forms of motor neuron degeneration that has been sub grouped into three categories due to age at onset, progression and the neuronal groups involved.

There is much heterogeneity within these three subdivisions of motor neuron disease such that the divisions are becoming blurred and it might be more advantageous to consider a spectrum of disease predominantly affecting motor neurons. The discovery of such mutations as VAPB that are causative for all types of MND further reduces the divisions.

Due to the size of the Brazilian family and number of affected members, the P56S mutation is almost certainly causative. However, when proposing a gene mutation as linked to a disease it is important to differentiate between simple polymorphisms and true causative mutations and having only one mutation, however large the family is not ideal. Recently however, a second mutation in VAPB has been found to be linked to cases of ALS and this greatly adds weight to the argument. J. de Belleroche identified a Threonine to Isoleucine substitution at position 46 (T46I) in a family unrelated to the original Brazilian family (personal communication). This and the original P56S mutation both fall within a stretch of amino acids that is highly conserved from yeast to man which add further strength to the importance of these VAPB mutations and makes it exceedingly unlikely that they are simply harmless polymorphisms.

1.9.1 Vamp Associated Protein of 33kDa (VAP-33)

Importantly in the quest to understand how this protein might lead to cases of ALS, some work on the characterization and function of human VAPB (hVAPB) as well as the homologues for this protein has already been carried out. In 1995 Skehel *et al* cloned a 33kDa protein from *Aplysia californica* and named it VAMP-associated protein 33 (VAP-33) for its ability to bind to the vesicle associated membrane protein synaptobrevin as discovered by yeast two-hybrid (Skehel *et al.* 1995). Injections of antibodies against this protein blocked neurotransmitter release in cultured neurons leading them to conclude that it plays a vital role in neuronal transmitter release. This lead to a number of other studies attempting to find a homologue for this protein in mammals and in 1998 Weir *et al.* identified a human homologue for the *Aplysia* VAP-33

(Weir *et al.* 1998). HVAP-33 shared an overall identity of 50% at the amino acid level with the highest similarity being at the N terminal with a region of 121 residues sharing 65% identity. The C-terminal, despite less comparable on a primary level, has a similar structural formation with a hydrophobic C-terminal and coiled-coil domains. Finally in 1999 the current knowledge that there are three human homologues was attained by Nishimura *et al* and these have been named VAP A, B and C. VAP A is the VAP-33 originally discovered by Weir *et al*. (1998). VAP B shares 60% homology to VAP A and 48% to a VAP-33. VAP C is a splice variant of VAP B and is missing a region 362 nucleotides in length. This induces a frame shift so that the transmembrane domain and coiled-coil domain present in A and B is not found in VAP C (Nishimura *et al.* 1999). In agreement with Weir, they found VAP A and B to be ubiquitously expressed. By comparing the VAP sequences for humans, *Aplysia* and rat which also has a VAP A and B homologue with a respective 95% and 88% identity to hVAPs, along with a putative homologue in *C.elegans*, Nishimura characterized the proteins and identified three conserved domains.

1.9.2 Structural and Phylogenetic Conservation of VAP proteins

All VAP proteins have a characteristic 3 domain structure. First a highly conserved amino acid motif CFKVKTTAPXXYCVRPNSG at position amino acid 41-59 is within a well conserved N-terminal region of the protein. Importantly it is the highly conserved proline at position 56 within this motif that is mutated in the ALS8 patients. The newly discovered T46I mutation is also within this conserved stretch of amino acids. Secondly there is a region with a number of conserved hydrophobic residues which make up the coiled coil domain and finally the transmembrane domain at the Carboxy terminal (Nishimura *et al.* 1999). See Figure 1.3 below.

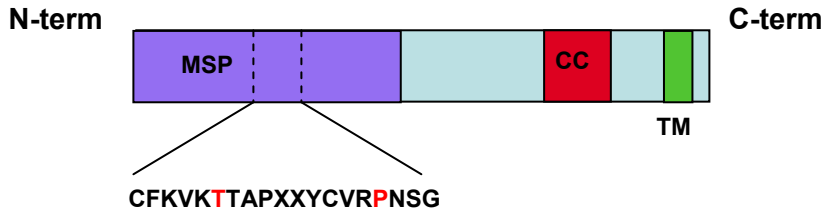


Figure 1.3 Schematic diagram of the conserved domains in VAP proteins
There are three conserved domains in the VAP family of proteins. The Major sperm protein domain (MSP) at the N-terminal, a coiled coil domain (CC) and the transmembrane domain (TM) at the C-terminal. Within the MSP domain is the highly conserved N-terminal motif containing the two mutated residues that have been linked to cases of ALS (highlighted in red).

The conserved N-terminal region of about 100 amino acids has been shown to share a high identity to the Major sperm protein (MSP) in the nematode *Caenorhabditis elegans* (Weir *et al.* 1998). The nematode MSP is found in the sperm cell and is necessary for the amoeboid locomotion adopted by these cells. Unlike the flagellum based swimming motion of sperm in other organisms, in nematodes the MSP forms non-polar filaments with two helical subfilaments wound round one another and the cyclical polymerization/depolymerization acts as a dynamic cytoskeleton that drives the sperm cells forward (Tarr and Scott 2005). In addition to this, the protein is also released from the sperm cells through a novel budding mechanism and acts as a signaling molecule to promotes oocyte meiotic maturation and gonadal sheath cell contraction (Kuwabara 2003). Spermatozoa do not contain the necessary ribosomes, ER and Golgi involved in the normal processing and release of proteins so it is believed that the MSP provides the force necessary for the protrusion of the membrane during budding (Kosinski *et al.* 2005). The signaling action of MSP is to antagonize the inhibitory somatic gonadal sheath cell pathway, in part, by acting on Ephrin (Eph) receptors on the oocyte (Kuwabara 2003). All VAPs in all organisms contain this MSP domain and the link between the cytoskeletal and signaling roles of the MSP and possible roles of this domain in VAPs has obviously been an area of much interest. So far, a lot of research has been done on the VAPs in various organisms and there is a both much similarity and variability in the function and believed roles of these proteins.

1.9.3 Yeast Scs2p

The yeast VAP homologue is called Scs2p. The gene *SCS2* was named for its identification as a multicopy suppressor of inositol auxotrophy of the choline sensitive mutant CSE1 and hac1/ire mutants (Nikawa *et al.* 1995; Kagiwada *et al.* 1998). Disruption of *SCS2* prevents yeast cells from growing well on plates lacking inositol in the medium as compared to various wild-type strains. Scs2p is a type II integral membrane protein and under normal conditions it is localized at the endoplasmic reticulum (ER). The protein is 35kDa in length which suggests post-translational modification because a direct translation from the transcript would produce a smaller peptide. Scs2p has a hydrophobic C-terminal of 16 amino acids corresponding to its transmembrane domain but unlike other VAP proteins it does not have a coiled coil domain (Kagiwada *et al.* 1998). A study looking for genes involved in telomeric silencing found *SCS2* to be a suppressor. In yeast, prevention of the transcription of the repetitive telomeric sequence at the end of a chromosome is ensured by a number of proteins, in particular Mec1p which regulates telomeric length and results in loss of telomeric silencing if disrupted. Overexpression of Scs2p could rescue this phenotype and null mutants also show a defect in telomeric silencing (Craven and Petes 2001). Unlike the role of VAP-33 in *Aplysia,* there seems to be no evidence of Scs2p having any necessary role in protein release.

1.9.4 *Caenorhabditis elegans* VPR-1

Not only does *C. elegans* have the MSP protein but there is also a homologue to the VAP-33 protein known as VAP33-related (VPR-1) (Tsuda *et al.* 2008). Disruption of this gene leads to problems in distal tip cell migration and the ventral hypodermal cell migration during enclosure. VPR-1 may act via a pathway involving Ephrin (Eph) receptors as a similar phenotype is seen when the Eph receptor VAB-1 is disrupted as

when VPR-1 is disrupted. As mentioned earlier MSP acts as a signaling molecule in *C. elegans* by binding to VAB-1 receptors on the surface of the oocyte and sheath cell plasma membrane. Interestingly the MSP domain of VPR-1 when removed and purified can promote the oocyte maturation and sheath cell contraction to a similar extent as MSP. Similar results were also found for the MSP domain of *Drosophila* and human VAP proteins. This indicates conservation of the function of VAPs as ligands in the Eph pathway (Tsuda *et al.* 2008).

1.9.5 *Drosophila melanogaster* DVAP-33A

Prior to the discovery of the role of VAP in ALS, the *Drosophila* homologue of VAP33 had already been discovered as DVAP-33A (DVAP). In *Drosophila* there are 3 potential homologues, the first with 39% identity is named *farinelli* but this is found exclusively in the testes. Second is DVAP-33B, but with only 18% homology it isn't the ideal candidate for the VAP-33 homologue. Finally there is DVAP with an identity of 40% and present on the X chromosome with a protein size of 35kDa (Pennetta *et al.* 2002). DVAP is localized both pre- and post- synaptically at the neuromuscular junction and is both associated with the membrane and found in the cytoplasm. It is enriched at the periactive zones which surround the active zones in the presynaptic boutons and shows an active relocalisation to those neurons undergoing budding (Pennetta *et al.* 2002). Further investigation into the role of DVAP by the production of null mutants and flies over-expressing VAP pre-synaptically found there to be a dosage dependent effect on the structure of the synapse. Loss of DVAP leads to a decrease in number of boutons but their size increases such that surface area remains the same. Likewise the over-expression of DVAP causes an increase in bouton number but they are smaller in size, again maintaining overall surface area. In *Drosophila,* as in mice, it seems that DVAP is associated with microtubules at specific points in the cytoskeleton network. It is proposed that through this interaction it mediates synaptic remodeling by changing the cytoskeleton organization at the synapse (Pennetta *et al.* 2002). It is

notable that in familial cases of ALS linked to SOD1 and Alsin and in these new VAP-linked cases, there is a common theme of cytoskeletal disruption (Pennetta *et al.* 2002; Takamiya *et al.* 2005; Hadano *et al.* 2006).

1.10 *Drosophila* model of ALS

Following the discovery of the link between VAPB and ALS, we mapped the human mutation equivalent in *Drosophila* to the Proline at position 58 and created a model of the disease by expressing the mutant P58S *DVAP* (*DVAPP58S*) in wild-type flies also expressing endogenous DVAP (Chai *et al.* 2008). As DVAP had previously been shown to have a role in synaptic homeostasis and considering ALS is a disease of the motor neurons, the neuromuscular junction (NMJ) of larvae was chosen as the model system to begin the characterization of mutant VAPs. The larvae musculature is highly stereotypic and has a regular pattern of 32 motor neurons per hemisegment that are accessible to electrophysiological readings and immunohistochemistry. It has been well characterized so any changes can easily be observed and recorded (Collins and DiAntonio 2007).

Prior to modeling the disease it was important to find out whether the human VAP was simply a homologue at the level of amino acid sequence or whether the two proteins were functionally interchangeable. We found that by expressing the human protein in a null back ground human VAP was able to rescue the mutant phenotype which indicates that at least some of the important pathways are conserved between species (Chai *et al.* 2008). Following on from this we examined whether DVAPP58S could also rescue the null phenotype as this would show whether or not the mutation is a loss of function. Interestingly both the *Drosophila* and human mutant proteins could rescue the null phenotype so it is likely that the mutation is either hypermorphic or neomorphic. A neomorphic mutation would mean that the mutant has gained a new toxic function unrelated to its normal wt role whereas a hypermorph has an excessive wt function.

Despite the apparent interchangeable nature of DVAP and hVAP, the *Drosophila* model was created by expressing the *Drosophila* mutant protein in a wt background. This would ensure that all the phenotypes witnessed are due to the mutation and not to a previously unseen incompatibility between the hVAP and the *Drosophila* system. Under these circumstances we created a model that recapitulated many of the major hallmarks of ALS including muscle wastage, denervation, locomotion defects and degeneration. Aggregates highly immunoreactive for DVAP were present in the nerve axons and cell bodies of the brain and this coincided with a decrease in DVAP staining at the nerve terminals. This suggests that the presence of mutant DVAP and the formation of aggregates is having a negative impact on the localisation of the wt protein and may be causing a situation similar to a dominant negative. Electrophysiological recordings were made at the NMJ and these showed a decrease in mini EJP (evoked junctional poteintial) amplitude; the response at the endplate to the release of a synaptic vesicle of transmitter. Possible explanations for this phenotype include a decreased sensitivity to the neurotransmitter or a reduction in the packaging of the vesicles. Although both are plausible, an observed change in the configuration and distribution of glutamate receptors indicates that the sensitivity of the receptors is being affected. This indicates a role for DVAP in the conformation of the post synaptic receptors at the NMJ which is very interesting considering mutant DVAP was only expressed pre-synaptically in the model. This implies that DVAP's effect is not cell autonomous and whether it triggers a pathway that crosses the synaptic cleft or VAP itself is secreted requires further research (Chai *et al.* 2008).

1.11 MSP secretion

The secretion of VAP or perhaps only the MSP domain is an intriguing idea considering the role of MSP in *C.elegans* as a signaling molecule. Tsuda *et al* 2008 found a protein cleavage product that compared in size to the MSP domain of DVAP in western blots prepared from protein extracts of *Drosophila* larvae expressing a tagged DVAP

transgene. They also found a similar product to be secreted from cells of the wing imaginal disc in cell culture. By tagging both the N-terminal and C-terminal of DVAP and expressing the protein within cell culture it was possible to see that the N-terminal was extra cellular and the C-terminal intracellular. A comparison of staining for the two tags showed that the N-terminal flag tag was more diffuse than the C-terminal HA tag still inside the cells of origin and only visible when the cells were permeabilised. This led them to conclude that the N-terminal part of the protein is being secreted and is free to move away from the cell. A similar experiment with DVAPP58S did not give the same results and showed no secretion suggesting that this mutation prevented the cleavage of the MSP domain. Similar to the results from Chai *et al,* DVAPP58S was found to form insoluble aggregates and they confirmed that they comprised both the mutant and wt protein (Tsuda *et al.* 2008). This is in keeping with the results from Chai *et al* showing that the expression of the mutant protein removed the wt DVAP from its normal localization into these insoluble inclusions.

The MSP of *C.elegans* acts through its interaction with the Eph receptor VAB-1. Injecting the MSP domain of either of the VAP homologues; VPR-1, hVAP or DVAP, into *C.elegans* all brought about oocyte maturation in a similar manner to endogenous MSP. The interaction with Eph receptor was further confirmed by co-immunoprecipitation (Tsuda *et al.* 2008). Eph pathways are involved in a number of cellular functions and this interaction with VAP gives us a new understanding of the possible roles played by this conserved family of proteins. Eph receptors in the fly are necessary for axon guidance and mutations cause loss of α lobes in the mushroom body. Similarly a loss of DVAP and double VAP/ Eph mutants also lack α lobes (Tsuda *et al.* 2008). Studies have found that Eph receptors are involved in the collapse of the growth cone through the control of the actin cytoskeleton via a number of Rho GTPases (Shamah *et al.* 2001). A recent screen looking for genes involved in the homeostasis of signaling at the synapse found Ephexin, a Rho-type guanine nucleotide exchange factor, to be required for the modulation of transmitter release. Ephexin is a known interactor of Ephrin receptors and a homozygous Eph receptor mutant or a heterozygous double mutant significantly disrupted synaptic homeostasis (Frank *et al.* 2009). Another study

found the mammalian EphB receptor and EphrinB to be involved in the formation of the synapse and necessary for the recruitment of NMDA receptors post synaptically (Dalva *et al.* 2000). Considering the involvement of DVAP in synaptic homeostasis and the cytoskeleton, the interaction with Eph receptors may hold the key for many of the roles played by the VAP proteins. The Eph receptor interaction shows how much more we can learn about a protein once we have an idea of the complexes it might form and the pathways in which it might be involved. As such a number of studies have been carried out to find other interactors of VAP proteins.

1.12 Known interactions of VAP and its homologues

1.12.1 Scs2p and its interacting partners

A comprehensive analysis of protein complexes of the yeast *S.cerevisiae* was carried out through the systematic purification of multiprotein complexes using tandem affinity purification (TAP) and mass spectrometry. 589 tagged proteins were purified and of these 78% were present with binding partners. One such protein was Scs2 and within the complex the following proteins were found.

Fks1 - FK506 sensitive-1 is an integral membrane protein of 215 kDa which is a subunit of the 1,3-b-D-glucan synthase (GS) (Mazur *et al.* 1995). The glucose polymer 1,3-b-D-glucan is a key component of the cell wall. Fks1 is closely related to Fks-2 and the two encode alternative catalytic subunits for 1.3-b-D-glucan synthase so there is a degree of redundancy. GS is necessary for cell wall remodeling and synthesis and an Fks1/2 double mutant completely shuts down cell wall synthesis. Fks1 localises to the plasma membrane at sites of cell wall remodeling and is the predominantly expressed protein of the two catalytic subunits (Mazur *et al.* 1995).

Num1 - Nuclear migration 1 is a 313kDa protein involved in coordinating nuclear movement into the yeast daughter bud through its interactions with dynein and tubulin. It has a Pleckstrin homology domain (PH domain) and is believed to act as an anchoring protein for dynein (Heil-Chapdelaine *et al.* 2000; Schauss and McBride 2007). In this way, a similar interacting protein of DVAP might exist that would account for the ability of DVAP to associate with microtubules and mediate synaptic budding. There is no record of a homologue for Num1p in terms of genetic sequence but it is likely that there is a functional homologue. Num1p is also involved in mitochondrial fission and is believed to provide a docking site for the dynamin related GTPase (Dmn1p) which is an inhibitor of mitochondrial division (Cerveny *et al.* 2007; Schauss and McBride 2007).

Opi1 – This is a transcription regulator of phospholipid synthesis and it acts by repressing the transcription of *INO1*. *INO1* is recruited to the nuclear membrane for transcription to be activated and Opi1 represses this. Scs2 is thought to work in conjunction with Hac1 by translocating to the nuclear membrane and stopping the reassociation of Opi1 to *INO1* (Kagiwada *et al.* 1998). An FFAT motif (two phenylalanines in an acidic tract) in Opi1 has been shown to be vital for the interaction with Scs2 (Loewen and Levine 2005).

Osh1 and Osh2 – Two of the 7 yeast homologues of the mammalian Oxysterol Binding protein (OSBP) involved in sterol metabolism. All 7 of the homologues have a conserved OSBP domain but only 1-3 have a Pleckstrin homology domain also found in OSBP. Osh1 and Osh2 also have three Ankyrin repeats at the N-terminal that are not present in the mammalian OSBP (Levine and Munro 2001). In yeast, the Osh family of genes have been shown to interact with Cdc42 (cell division cycle 42) which is a member of the Rho family of GTPases involved in cell polarity and morphology. Osh proteins also affect the localisation of another Rho GTPase, Rho1p, and are involved in the delivery of vesicles to the bud tip for polarized secretion (Kozminski *et al.* 2006). Studies have also shown the *Osh* family to be necessary for the internalization step of endocytosis (Beh and Rine 2004).

Rpn10 – Non ATPase base subunit of 19s regulatory particle of the 26s proteosome. The yeast Rpn10 is necessary for the targeting of ubiquitinated substrates to the proteasome for degradation. Through the degradation of proteins such as cyclins and p53 the proteasome is involved in the control of the cell cycle and proliferation as well as in the degradation of damaged proteins by proteolysis (Glickman *et al.* 1999). There is a degree of redundancy for ubiquitin binding factors in yeast as Rpn10 null mutants are viable (Madura 2004).

Stt4 – Phosphatidylinositol-4-kinase necessary for the production of PI(4)Ps. It was identified in a screen for hypersensitivity to stauroporine, an inhibitor of Protein Kinase C. Yeast Stt4 mutants present with defects in vacuole morphology, cell wall integrity and actin cytoskeleton. Interaction with Sfk1, an integral membrane protein, is necessary for its localisation to the plasma membrane where it controls the levels of PI(4)P which are thought to have their own signaling functions as well as act as a substrate for $PI(4,5)P_2$ production. PI(4)P levels have been recently shown to have their own signaling properties and directly affect vacuole morphology (Foti *et al.* 2001). The production of PI(45)P2 and activation of the Rho1/PKC1-mediated Map kinase cascade are necessary for the other cellular functions affected in the Stt4 mutants (Foti *et al.* 2001; Audhya and Emr 2002).

Swh1 –Initially characterized as a single gene the open reading frame for Swh1 is now realized to be part of the full open reading frame of Osh1. It encodes the 5' region which includes the Ankyrin repeats (Levine and Munro 2001).

As the conservation of VAP proteins between species has been so strongly maintained so might we expect to find homologues for the interacting proteins described above in other more complex organisms. Already a number of interacting partners of the mammalian VAP have also been discovered largely through the appearance of VAP as a binding partner of another protein of interest. A brief account of some of these binding partners is given below.

1.12.2 Human VAPA interacts with Occludin

The human VAP-33 protein (VAP A) was picked out in a yeast 2-hybrid screen aimed at looking for binding partners of Occludin, a transmembrane protein located at tight junctions (Lapierre *et al.* 1999). These specialized junctions are found between epithelial and endothelial cells and they create a tight seal between cells and establish a polarity between plasma membrane domains. Occludin has a role in cell cell adhesion and in the formation of the paracellular barrier. It is also involved in signal transduction as it has been shown to activate the small GTPase, RhoA, and alter the actin cytoskeleton. In another study it was shown to inhibit apoptosis via activation of the mitogen activated protein kinase (MAPK) and Akt pathways (Chiba *et al.* 2008). Following the identification of VAPA as an interactor, the co-localisation of the two proteins was investigated. There appeared to be two populations of VAP in epithelial cells, one being cytoplasmic, the other localized at the tight junction and co-localised with Occludin (Lapierre *et al.* 1999). It is yet to be determined if this interaction is conserved in other species and whether DVAP is also localized at the equivalent of the tight junction in flies termed the Septate junction (Woods *et al.* 1996).

1.12.3 The VAP - FFAT interaction and the Golgi membrane

A group of proteins found to interact with VAP are involved in the biosynthesis and trafficking of lipids and sterols and are linked by a common motif termed the FFAT motif. This is so called for the presence of two phenylalanines within a conserved acidic tract and it is through this domain that these proteins interact with VAP and are localised to the ER. The three members of this group are the Oxysterol Binding Protein (OSBP), Ceramide Transfer protein (CERT) and Nir2 and they are all located at the Golgi and ER and translocate between these two cellular organelles. The presence of VAP at the ER

directly affects the localisation of all these proteins and as such VAP is a key player in the roles played by these lipid trafficking and sterol sensing proteins. The current knowledge of what these roles are, are outlined below.

1.12.4 Nir2

Nir2 is a phosphatidylinositol/phosphatidylcholine PI/PC transfer protein involved in the regulation of Diacylglycerol (DAG) levels at the Golgi apparatus through the inhibition of the CDP choline pathway.(Litvak *et al.* 2005) DAG is necessary for protein transport from the Golgi to the plasma membrane through its crucial role in the fission of transport carriers at the trans Golgi network (TGN). Nir2 has also been implicated in cell morphology and is necessary for the completion of cytokinesis through an interaction with the small GTPase RhoA. (Litvak *et al.* 2002) In mammals there are three Nir proteins, Nir1-3, all belonging to a well conserved family Nir/rdgB involved in regulating phospholipid trafficking, metabolism and signaling. The *Drosophila* homologue is the Retinal degradation B (rdgB) protein characterized for its role in photo-receptor cell viability and light response. RdgB, Nir2 and Nir3 contain an N-terminal PI transfer domain that is necessary for the transfer of PI and PCs between membrane bilayers. Nir2 is predominantly localized at the Golgi apparatus but there is some localization at the ER and this is thought to be accountable to the interaction with VAP (Litvak *et al.* 2002; Amarilio *et al.* 2005). Amarilio *et al* discovered the interaction of the Nir proteins with VAPB using co-immunoprecipitation and GST pull down experiments. They characterized the interaction as occurring through the conserved FFAT motif present in Nir1-3, of the sequence EFFDAXE. Co-expression of VAP and Nir2 in HeLa cells lead to changes in the ER structure and the creation of stacked membrane arrays which was dependent upon the presence of the FFAT motif and did not occur following over expression of either protein alone. The exact mechanism for this stacking phenomenon is unknown but a few hypotheses implicate the ability of VAP

proteins to oligomerize and bridge between the membranes following a conformational change induced by Nir2.

1.12.5 Oxysterol Binding Protein (OSBP)

An interaction between VAP and OSBP has been shown in all organisms from yeast to man. OSPB binds oxysterols, the hydroxylated derivative of cholesterol, and in mammals it is believed to play a role in lipid metabolism and inhibition of the mevalonate pathway (Alphey *et al.* 1998; Lehto and Olkkonen 2003). However, insects are unable to biosynthesize sterols and have to take them in as part of their diet so it is likely that there is a separate role for OSBP in the insect that is also conserved in humans. A number of studies have focused on the yeast homologues of OSBP, Osh1-7p. Interestingly, considering the effect that DVAP has on the morphology of synaptic boutons, Osh3p is involved in the control of membrane growth in yeast (Lehto and Olkkonen 2003).

Furthermore the Osh proteins have been shown to interact with Cdc42 (cell division cycle 42), a member of the family of Rho GTPases (Kozminski *et al.* 2006). This interaction is important considering that the Rho GTPases play a number of roles in the establishment and maintenance of cell polarity and morphology. In *Drosophila* Cdc42 activity is necessary for the final stages of dorsal closure through the induction of filopodia during this morphogenesis (Van Aelst and Symons 2002). A conservation of the interactions of OSBP would be interesting as Cdc42 signaling is also involved in microtubule growth and actin filament assembly and the family of Rho GTPases have been shown to act on the neuronal cytoskeleton and affect dendrite and spine morphology (Newey *et al.* 2005).

In yeast the *Osh* genes were found to affect the localization of Cdc42p and Rho1p and as such will affect the various pathways through which these GTPases act (Kozminski *et al.* 2006). Nothing is known about the role of OSBP at the synapse but if it is a significant interactor of DVAP, as concluded from a number of yeast two-hybrid

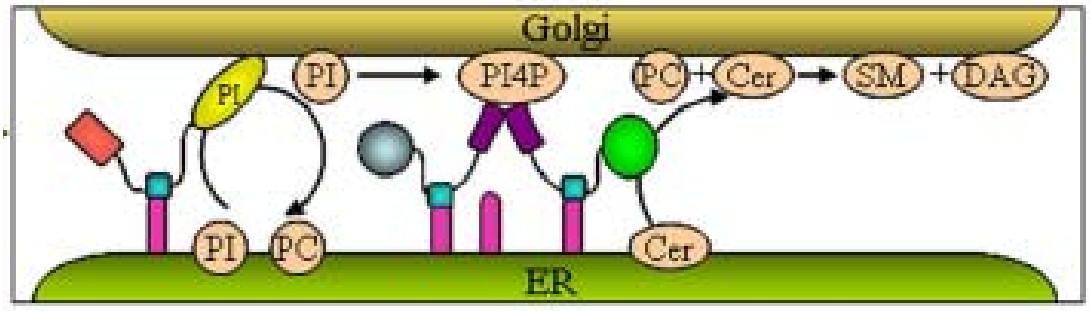
studies, it is likely to be involved in synaptic morphogenesis and it could be a key player in the pathways through which DVAP acts (Wyles *et al.* 2002; Giot *et al.* 2003). OSBP and its yeast homologues Osh1-3 contain a Pleckstrin Homology (PH) domain which is necessary for the localization to the Golgi network by mediating an interaction with PI(4)P present in the Golgi membrane (Levine and Munro 2001; Xu *et al.* 2001). Mutations in the PH domain of OSBP increased the interaction with human VAP A as evident by the increased localization to the ER. This might be due to a reduced interaction with the Golgi network or may be that the mutation increases the propensity for the VAP interaction (Perry and Ridgway 2006). In the same study, OSBP was also found to directly affect the localisation of CERT. Under conditions which induce an upregulation of Sphingomyelin synthesis, including the addition of exogenous 25-hydroxycholesterol (25OH), a translocation of OSBP and CERT to the Golgi was observed where these two proteins then colocalized. Upon reduction of OSBP, or any of its FFAT, PH or Oxysterol binding domains, the translocation of CERT was no longer witnessed and as such the transportation of Ceramide is inhibited. This in turn stopped the production of Sphingomyelin and DAG from the substrates Ceramide and PC. It is now believed that OSBP functions as a sterol sensor and is necessary for the activation of CERT at the Golgi. A closer look at CERT by Perretti *et al* further uncovered the interconnecting pathways within this group of VAP interacting proteins.

1.12.6 Ceramide transfer protein (CERT)

ER-Golgi membrane contact sites are required for non-vesicular ceramide transport via the ceramide transfer protein CERT (Hanada *et al.* 2007). CERT interacts with the Golgi network through its PH domain which binds to the Phosphatidylinositol-4-phosphate (PI4P) present at the Golgi. It also binds to ER through the FFAT motif and this is via VAP A and VAP B (Kawano *et al.* 2006; Peretti *et al.* 2008). Ceramide is produced from L serine and Palmitoyl Co A by a number of reactions at the cytosolic

surface of the ER. It is then transported to the Golgi where it can be converted to sphingomyelin (SM) and DAG along with other complex glycosphingolipids (Hanada *et al.* 2009).

Peretti *et al* examined the role of the VAP-CERT interaction at the ER by knocking down the level of VAP through small interfering RNA (siRNA) in HeLa cells. SiRNA triggers the degradation of homologous mRNA, in this case VAP, and in doing so effectively silences the gene. Expression and localization of CERT along with Nir2 and OSBP were recorded and although expression did not alter there was a significant increase in cytosolic localization of the VAP interacting proteins and a loss of Golgi targeting (Peretti *et al.* 2008). The Golgi localization is normally maintained by the interaction of PH domain of OSBP and CERT with PI4P and a reduction of VAP was found to directly affect the levels of key lipids including PI4P, DAG and SM. Interestingly the expression of Nir2 at a high level could compensate for the reduction in VAP and restored the localization of OSBP and CERT at the Golgi. This was accounted for by the possibility that over expression of Nir2 at such a high level could bypass the need for a binding partner in the form of VAP at the ER. Even without VAP, Nir2 could carry out its normal function which is to transport PIs from the ER to the Golgi where they are available for production of PI4P. This then allowed for the re-targeting of OSBP and CERT to the Golgi. In this way, all three of the FFAT/VAP interacting proteins are linked as illustrated in figure 1.4. VAPs presence at the ER is necessary for the binding of Nir2, CERT and OSBP. If VAP localises to the ER-Golgi membrane contact sites during periods of reduced cholesterol synthesis, Nir2 would too be localised here where, through its N-terminal PI/PC transfer domain it exchanges PIs from the ER for PCs following the concentration gradient. This allows for the production of PI(4)P at the Golgi which acts as an anchor for OSBP and CERT and through their colocalisation, CERT is activated to bring about the transport of ceramide to the Golgi. This then leads to the production of SM and DAG. The removal of PCs from the Golgi by Nir2 also inhibits the CDP choline pathway and prevents the degradation of DAG (Peretti *et al.* 2008).



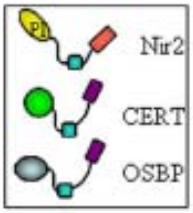


Figure 1.4 Diagram illustrating the possible interacting pathways of CERT, Nir2, OSBP and VAP at the ER-Golgi contact sites.
Vap (in pink) provides an anchor for the FFAT containing proteins at the ER. (Peretti *et al.* 2008)

A number of in vivo studies have been carried out to look closer at the function of CERT. A *Drosophila DCERT* knock out has been created and although the flies are viable they have a significantly shortened life span and decreased ceramide and CPE (sphingomyelin equivalent) levels (Rao *et al.* 2007). The reduction in CPE caused an increased fluidity of the plasma membrane and this made the cells more vulnerable to oxidative stress as seen by an increase in lipid peroxide products. Aging flies showed metabolic and motor defects but there was no evidence of neuronal degeneration. The overall phenotype was similar to that of accelerated aging (Rao *et al.* 2007). In mice lacking *CERT,* homozygous mutants die as embryos at E11.5 (Wang *et al.* 2009b). Similarly to *Drosophila* they have a decreased level of sphingomyelin but there is no difference in membrane fluidity, perhaps because the decrease is only 50% as compared to >70% in flies (Rao *et al.* 2007). The level of ceramide in the mutant mice was normal but the localisation was altered with an increased concentration at the ER. Examination at a cellular level using transmission electron microscope (TEM) found the ER to be engorged and also the mitochondria were abnormal, some degenerating. The change in ER morphology taken together with an observed increase in various UPR pathway components indicated that the mice were undergoing chronic ER stress. A number of

pro-apoptotic factors were also examined but showed no change as compared to wild type controls. There was a significant increase in ceramide levels in the mitochondria and this had a negative impact on the activity of cytochrome c oxidase, the terminal enzyme of oxidative phosphorylation. The final cause of death in the mice was due to a failure to establish a functioning cardiovascular system due to defects in cell cycle and differentiation but not as a result of apoptosis (Wang *et al.* 2009b).

Despite the apparent lack of apoptosis in the model created by Wang *et al* (2009), ceramide has previously been shown to induce apoptotic cell death when expressed in a range of neuronal cell types including motor neurons (Irie and Hirabayashi 1998). At a low level however, it has a protective role against apoptosis. Alzheimers patients have shown an increase in ceramide levels along with cholesterol and it is believed that it is involved in the activation of degeneration although perhaps through a mechanism other than apoptosis as there is a lack of caspase activity (Marks *et al.* 2008). Ceramide is also thought to be involved in the formation of atherosclerotic plaques and is a key signaling molecule in stroke (Adibhatla and Hatcher 2008). There is a rare autosomal recessive disease called Farbers disease which is caused by mutations in the acid ceremadise gene which results in an accumulation of ceramide in cells. Patients suffer with progressively deformed joints, subcutaneous nodules and premature death (Levade *et al.* 1995; Bar *et al.* 2001).

Taking all of the above into account, the interactors of VAP have a number of important cellular functions requiring a tight regulation of their localisation and production. Mutations affecting VAP, a key player in regulating the localisation of these proteins, will presumably have a number of negative effects on the cell and this may be involved in the phenotypes associated with ALS8. There is still much to learn about the overall role played by VAP proteins in the cell and the aim of this project is to move a few more steps towards a clearer picture of what that might be.

1.13 Aims

Through the described interactions, VAP has a number of important roles in the cell including membrane lipid composition, cytokinesis and cell morphology. Add to this the data from *Drosophila* implicating DVAP in the formation of boutons and the glutamate receptor configuration, it is obvious that the VAP proteins are key integral membrane molecules. It is no wonder that the mutation discovered has a serious effect on the cell but what is still not clear is why it is the motor neurons that are affected in the ALS 8 patients and why there is a late onset degenerative phenotype. For this reason, research into the function of VAP proteins is still vitally important as the more knowledge gained, the greater will be our understanding of the problems that might arise when the mutation is present. The aims of my thesis are all geared towards increasing our understanding of VAP and discovering how the mutation might affect the normal functioning of this protein.

Aim 1. Characterisation of the VAPT46I mutation. In order to characterize the new mutant protein the equivalent mutation will be mapped in DVAP and a mutant transgene expressed in *Drosophila.* The resulting phenotype will be compared to the DVAPP56S model of disease.

Aim 2. Verification of the homodimerization of VAP proteins and the effect of the mutations. The *Drosophila* VAP protein is known to homodimerise and as the human homologue VAPB is similar in structure and has the ability to rescue the knock-out fly, it would be expected that it has many of the same biochemical properties. This will be tested by yeast two-hybrid screening to examine the interaction between the VAP proteins.

Aim 3. Confirmation of VAP-SAC Interactions. Sac1 was shown to be a potential interactor of DVAP in a large scale yeast-two hybrid screen of the protein interactions of

Drosophila (Giot *et al.* 2003). Considering that FIG4, also known as SAC3, has been linked to cases of ALS and it too is a lipid phosphatase this is potentially a very important interaction. This interaction will be verified by small scale yeast two-hybrid looking at the binary interaction of *Drosophila* VAP and the *Drosphila* Sac1. As a functional homologue, human VAP should also be able to form the same complexes as its *Drosophila* counterpart. Therefore, if the interaction is important, this could mean that the interaction with Sac1 is conserved despite the species difference.

Aim 4. Examining the role of Sac1 in the fly. After studying the biochemical properties of Sac1, an investigation of the protein in the fly will be examined in two ways. Overexpression of the protein by utilizing the GAL4/UAS system or removal of the gene to create a knock out will hopefully produce some interesting phenotypes that will expose the pathways in which it is involved. Already Wei *et al.* (2003) have shown that Sac1 is involved in the closure of the amnioserosa during gastrulation as a full null mutant is reported as embryonic lethal (Wei *et al.* 2003a). If this is the case a hypomorphic allele may be used to overcome this lethality whilst still giving a phenotype that will allow the characterization of Sac1 at the synapse.

Aim 5. Confirmation of VAP/CERT interactions. The interaction of VAPs with the FFAT domain proteins is potentially very important to understanding the pathogenesis of disease in ALS8. Confirmation of the interaction with dCERT, a known FFAT domain protein in *Drosophila,* and the effect of the mutation on this interaction will increase our underatanding of the impact of VAP mutations on downstream pathways

Aim 6. The identification of DVAP interactions by a library scale yeast two-hybrid screen. Using a *Drosophila* embryonic cDNA library the *Drosphila* genome will be screened for potential interactors of DVAP using the yeast two-hybrid system. The interacting genes will be identified by sequencing and then the interaction verified by small scale yeast two hybrid using full length cDNAs as many of the library cDNAs are truncated C-terminal fragments. Co-IP will again be used to verify the interaction.

Chapter 2: Material and Methods

2.1 *Drosophila melanogaster* stocks

Strain	Source
Canton S	Bloomington *Drosophila* Stock Center
elav^{C155}*-Gal4/elav*^{C155}*-Gal4;+/+*	Bloomington *Drosophila* Stock Center
yw/Y; +/+; *ey-Gal4/ey-Gal4*	Bloomington *Drosophila* Stock Center
BG57-Gal4	(Budnik *et al.* 1996)
yw/yw; UAS-DVAP/UAS-DVAP; +/+	G. Pennetta
yw/yw; UAS-DVAPP58S/UAS-DVAPP58S; +/+	G. Pennetta
yw/yw; UAS-DVAPT48I/UAS-DVAPT48I; +/+	G. Pennetta
yw/yw; UAS-Sac1RNAi/UAS-Sac1 RNAi; +/+	Bloomington *Drosophila* Stock Center
yw/yw; UAS-SAC1/UAS-SAC1;+/+	G. Pennetta

Table 2.1 *Drosophila* Stocks

2.2 Primary Antibodies

Primary Antibody	Source
AffiniPure Rabbit α-HRP	Jackson ImmunoResearch
Guinea Pig α-DVAP	G. Pennetta
Rabbit α-hVAPB	Sima Lev
Rabbit α –c-Myc	Sigma
Mouse α -Flag	Sigma
Rabbit α –SERCA	Sanyal *et al* 2005
Mouse α -KDEL	Stressgen
Mouse α –HSP70	Affinity Reagents

Table 2.2 Primary Antibodies

2.3 Secondary Antibodies

Antibody	Source
Goat α-rabbit Biotinylated IgG	Vector Laboratories
Goat α-rabbit FITC (Flourescein isothiocyanate)	Jackson ImmunoResearch
Goat α-rabbit HRP	Jackson ImmunoResearch
Goat α-guinea pig HRP IgG	Jackson ImmunoResearch
Goat α – mouse Cy3 (Cyanine)	Jackson ImmunoResearch
Goat α –rabbit Cy3	Jackson ImmunoResearch
Goat α –guinea pig Cy3	Jackson ImmunoResearch

Table 2.3 Secondary Antibodies

2.4 General Cloning Procedure

2.4.1 Polymerase Chain Reaction (PCR)

The following reagents were mixed in a 0.5ml PCR tube on ice; 5µl Pfu 10x reaction buffer (Stratagene), 3µl template DNA (300ng), 5µl forward primer (10µM stock), 5µl reverse primer (10µM stock), 5µl dNTP (10mM stock), 26µl ddH_2O and 1µl Pfu polymerase enzyme (Stratagene). The reaction mixture was overlaid with 50µl mineral oil and the tube inserted in a thermocycler set with the following program. 1) One cycle of 95^{o}C denaturation for 2 min, 2) 30 cycles of 95^{o}C denaturation for 30 sec, 60^{o}C annealing for 50 sec and 68^{o}C extension for 2 min, 3) Final cycle of 68^{o}C extension for 10 min. When complete, the DNA solution was removed from below the mineral oil and cleaned by phenol extraction (200µl Phenol Chloroform/ 200µl Tris EDTA (TE, pH8.0) and precipitated (20µl 3M NaAc/ 500µl 100% Ethanol) overnight at $^{-}$20^{o}C or for 1 hour at $^{-}$80^{o}C.

2.4.2 Restriction Digest

Precipitated DNA was resuspended in TE pH 8.0. Restriction enzymes (New England Biolab), specific for restriction sites within the primers (see table 2.4) and the multiple cloning site of the vector, were either used sequentially or combined depending upon their compatibility. If individual digestions are required the DNA was resuspended in 23µl TE and the reaction set up as follows in a single ependorf; 23µl DNA, 3µl 10x BSA, 3µl 10x restriction buffer and 1µl restriction enzyme (all solutions from New England Biolab). The reaction was then incubated at 37^{o}C for 2 hours. The same digestions are carried out for the vector DNA. If two enzymes are compatible the total volume is increased to 40µl and the 2 enzymes added together (30µl DNA, 4µl BSA, 4µl buffer, 1ul enzyme1 and 1µl enzyme 2) and incubated for 4 hours at 37^{o}C. Following the digestion reaction, the DNA was again cleaned by phenol extraction and precipitated overnight.

2.4.3 Ligation

Prior to ligation the precipitated insert DNA was resuspended in 17µl TE (pH8.0) and the vector resuspended in 10ul. 1ul from each is then run on a 0.8% agarose gel so that a rough estimate of DNA ratio can be made. If necessary the vector DNA was further diluted to optimize the probability of insert being taken up by the vector. The following reaction is then set up and put at 14^{o}C overnight. 16µl insert (resuspended in TE pH8.0), 1µl vector, 2µl l0x ligase buffer, 1µl DNA ligase (Promega)

2.4.4 Transformation

Chemically competent bacterial cells XL1-blue (Stratagene) were used and transformed with the ligated DNA constructs. To 100µl cells 1.7ul B-ME was added and the cells incubated on ice for 10 minutes. Then 10µl of DNA ligation mixture was added to the cells and incubated for a further 30min on ice. Mean while the SOC broth was prepared and preheated and then the reaction was heatpulsed at 42^{o}C for 45 seconds and then incubated on ice for 2 min. 1ml SOC broth was added to the cells followed by a 60 min recovery incubation at 37^{o}C with shaking. 200ul of mixture was added per plate containing the necessary antibiotic, Kanamycin, Ampicillin of Chloramphenicol depending on the resistance conveyed by the vector. Plates were incubated overnight at 37^{o}C.

2.4.5 Amplification/extraction of DNA by mini prep (boiling method)

DNA was harvested by transferring single bacterial colonies into 2ml of LB medium containing the appropriate antibiotic in a 5ml bijou tube incubated overnight at 37^{o}C with shaking. 1.5ml of the culture was then transferred into a microcentrifuge tube and centrifuged at 12,000g for 2 min. The supernatant was removed by aspiration and the bacterial pellet resuspended in 350ul STET, mixed by vortexing and then 25ul of freshly made lysozyme (10mg/ml in 10mM Tris Cl, pH8.0) added. The microcentrifuge tubes are placed in a boiling waterbath for 1min then centrifuged for 10 min at 12,000g. The pellet of bacterial debris was removed using a toothpick and to the remaining supernatant 40ul NaAC (2.5M, pH5.5) and 420ul Isopropanol was added to precipitate the DNA. The reaction was mixed by vortexing briefly, storing at room temperature for 5 min and then centrifuging for 10min at 12,000g. The supernatant was removed by aspiration and the pellet of DNA washed in 70% Ethanol. This was again centrifuged

for 2 min at 12,000g and then the supernatant removed by aspiration. Once dry, the pellet was resuspended in 50ul TE pH8.0.

2.4.6 Clean preparation of DNA

Restriction digests were carried out to identify clones carrying the correct insert and 200ul of the corresponding bacterial culture used to inoculate 150ml LB and incubated in a 500ml conical flask overnight at 37^{o}C with shaking. The DNA was extracted using a Qiagen maxiprep kit which uses anion-exchange resin in QIAGEN-tips to selectively bind plasmid DNA and allow contaminating impurities to be washed away leaving the ultrapure plasmid DNA to be eluted in high salt buffer. The resulting DNA pellet was resuspended in 300ul TE (pH8.0).

2.5 Site directed mutagenesis

In vitro site-directed mutagenesis was carried out using the QuikChange II XL site-directed mutagenesis kit from Stratagene. CDNA was obtained for SAC1 from the Drosophila Genomics Resource Centre (DGRC) in the form of clone GH08349 in pOT2 vector. This was first subcloned into the smaller vector pBluescript SK+ using primers aligning to the sequence upstream and downstream of the open reading frame to amplify the Sac1 cDNA by PCR. **5'Sac1 (**5'-TATTGGATCCCCATCCCTATCACACACG) **3'Sac1**(5'GAGCGGTACCCTAGCA ATGCGTAAAATA) following the general cloning procedure outline above and using BamH1 and Kpn1 restriction enzymes. The following primers were then used in order to carry out the deletion and substitution (D to N) mutagenesis of *SAC1*.

Deletion 5' (5'-GCAGACTGGTGTCTTCGTCGTGCAGAGCATGC)

3' (5'-GCATGCTCTGCACGACGAAGACACCAGTCTGC)

D to N 5' (5'-CTTCCGAACGAATTGTATCAATTGTCTCGATAGGACGAACG)

3' (5'-CGTTCGTCCTATCGAGACAATTGATACAATTCGTTCGGAAG)

For DVAPT48I mutagenesis the DNA template was in the form of LD06870 also obtained from the DGRC, which is already in a pBluescript vector. The primers were as follows;

DVAPT48I sense (5'-TCTGGTCTTCAAGATCAAGATAACCGCCCCGAA)

DVAPT48I antisense (5'-TTTCGGGGCGGTTATCTTGATCTTGAAGACCAGA)

The QuikChange II XL site-directed mutagenesis kit from Stratagene uses PCR to replicate the whole plasmid while adding the desired mutation. In the case of the deletion, the primers span the deleted region and because a large fragment was to be removed, an extra step of PCR had to be carried out to ensure that the primers anneal to the template DNA and not to each other. The primers for the single Sac1 D to N mutation and the DVAPT48I mutagenesis complement the template enough to make this extra step unnecessary.

For the deletion mutagenesis the following reaction was first set up with two tubes, one for each primer (sense and antisense). 5ul of 10x reaction buffer, 3ul of DNA template (50-200ng), 1ul primer (5' or 3'), 1ul dNTP, 3ul Quik solution, 37ul of H_2O and 1ul Pfu DNA polymerase. The reaction was overlaid with 50ul mineral oil and placed in the thermocycler for the following program; 1) One cycle of 95^{o}C denaturation for 30 sec, 2) Ten cycles of 95^{o}C denaturation for 30 sec, 55^{o}C annealing for 1 min and 68^{o}C extension for 8 min. 25ul was then removed from below the mineral oil of each of the two reactions and added together in a separate tube. The second step and the only necessary step for the other constructs are as follows. After mixing together all the reaction solutions including both primers and 1ul of Pfu polymerase, the PCR was carried out with the following program. 1) 95^{o}C denaturation for 1 min 2) 18 cycles of 95^{o}C denaturation for 50 sec, 60^{o}C annealing for 50 sec and 68^{o}C extension for 6 min, 3) One cycle of 68^{o}C extension for 7 min.

To remove the template DNA 1ul Dpn I was added to the reaction mixture below the mineral oil and the reaction incubated for 1 hour at 37^{o}C. This should digest all non mutated supercoiled DNA as Dpn I is an enzyme specific to methylated DNA. The PCR product is not methylated and as such is not digested. The next step was to

transform supercompetent cells with the mutant DNA. XL10 Gold ultracompetent cells are provided with the kit and are made competent by the addition of B mercaptoethanol (B-ME). To 45ul cells 2ul B-ME was added and incubated on ice for 10 minutes. Then 5ul of mutant DNA mixture was added to the cells taking care to remove residual mineral oil and incubated for a further 30min on ice. Meanwhile NZY^+ broth was preheated and the reaction then heat pulsed at 42^oC for 30 seconds followed by 2 min incubation on ice. 0.5ml NZY^+ broth was added followed by a 60 min recovery period at 37^oC with shaking. The mixture was then divided into two and plated on LB agar medium containing Ampicillin. After an incubation of 16 hours, a number of colonies were picked and cultures prepared and grown overnight in LB broth containing Ampicillin to amplify the DNA. Qiagen miniprep was carried out following manufacturer instructions to obtain clean DNA suitable for sequencing by the Dundee Sequencing Service.

Positive clones were obtained and the final step was to subclone the mutated Sac1 and DVAP into the *Drosophila* transformation vector pUAST for injection into the fly. The desired Sac1 fragment was removed from pBluescript by restriction digest using BamH1 and Kpn1 (NEB) sequentially. The fragment of DNA was then separated and purified by running the DNA on an agarose gel and carrying out a Qiagen gel extraction following manufacturers guidelines. The recovered insert DNA was then ligated into the multiple cloning site of pUAST cut with BglII and Kpn1 which provided the necessary overhanging ends for the ligation. DVAPT48I insert was amplified and removed from pBluescript by PCR and then ligated into pUAST.

2.6 *UAS-SAC1* Cloning Strategy

The GAL4/UAS system is used in *Drosophila* to target the expression of genes and the pUAST vector is used to insert a UAS-gene into the *Drosophila* genome. This is a vector into which genes can be subcloned downstream of a tandem array of five optimized GAL4 binding sites (Upstream Activation Sequence, UAS), and an Hsp70

TATA box and transcriptional start. The polylinker contains 6 unique sites and is followed by a SV40 small-t intron transcriptional terminator and polyA site. The vector was created by inserting this fragment into the P-element vector pCaSpeR3 which contains P element ends (P3' and P5') and the white gene thus enabling the insertion of the UAS-gene into the genome. The UAS-gene remains silent unless the fly is crossed with a fly expressing GAL4 (Enhancer trap GAL4) and then it is only expressed in those cells expressing the GAL4.

The full length transcript for Sac1 was cloned into pUAST from the GH08349 clone. The following primers were used to remove and amplify the sequence from the pOT2 vector by PCR following the procedure described in section 2.4

5'Sac1 (5'-GATCAGATCTCCATCCCTATCACACACG)

3'Sac1 (5'-GAGCGGTACCCTAGCAATGCGTAAAATA)

In the above oligonucleotides the 5' primer introduced a unique BglII site upstream of the sequence, the 3' primer introduced a unique Kpn1 site at the end of the sequence. Once a positive clone was obtained and a clean maxiprep stock made the vector was injected in *Drosophila*.

2.7 Injection Protocol

The injection of clones into flies was carried out in our lab by Andrea Chai following the basic protocol outlined here. Delta 2-3 flies were amplified on grape juice plates with yeast paste and on the morning of injection, the plates were changed every 30 minutes. 3ul of the DNA diluted in TE was loaded into the needle of the injection microscope and the compensation pressure set to ensure that during injection, medium will not flow into the needle from the cell. Once there are enough embryos laid during the 30 minute period the embryos are carefully washed from the plate into a mesh basket using milliQ water and then they are dechorionated using a 50% bleach solution. The basket was held in this solution for 3 minutes with agitation then washed clean with milliQ water and spread onto a piece of red grape juice agar. The embryos were carefully aligned against

the edge of the agar with the micropile facing outwards and then stuck carefully onto a coverslip wrapped in double sided tape. The coverslip was placed onto a slide and the embryos desiccated by placing in a chamber with silica gel for approximately 4 minutes depending on room humidity. The embryos were then covered in a drop of heavy oil and were ready to be injected into the germ-line precursor cells which must be done before cellurization occurs. The resulting surviving larvae were carefully placed in a vial of food and the resulting flies crossed as virgins to yw (yellow white) flies. The F_0 are phenotypically normal but the resulting F_1 from these crosses may be transgenic. Pigmentation in the eye indicates the single or multiple insertion of the transgene because of the presence of the linked mini-white reporter gene.

2.8 Immunohistochemistry

2.8.1 DVAP staining of third instar larval NMJs

Wandering third instar larvae were selected and dissected in 1xPBS (phosphate buffered solution) and then fixed in Bouin's fixative (15:5:1 saturated picric acid, 37% formaldehyde and glacial acetic acid) for 5 minutes. The samples were washed in 0.1% PBT (PBS + 0.1% TritonX-100) and then blocked with NGS for 30 minutes under rotation. The antibodies; Affinipure rabbit α-HRP (Horseradish peroxidase) (Jackson ImmunoResearch) and guinea pig α-DVAP (GP33) (Pennetta *et al.,* 2002) were then added in 5% NGS (normal goat serum) and 0.1% PBT at a concentration of 1:500 and 1:1000 respectively and the samples put at 4^{o}C to rotate overnight. The samples were washed for 2 hours in 0.1%PBT, changing the solution every 15 minutes, and then incubated with the secondary antibodies; α rabbit FITC (flourescein isothiocyanate) (Jackson ImmunoResearch) and α GP-Cy3 (cyanine) at 1:1000 and 1:500 respectively in 5% NGS and O.1% PBT. After 2 hours incubation at room temperature the samples were again washed for 2 hours in 0.1% PBT and then mounted on a slide with

Vectashield (Vector Laboratories). Larval NMJs were imaged using an Axiovert Zeiss microscope.

2.8.2 Staining of third instar larval brains

Larvae were dissected in 1x PBS and fixed in Bouin's fixative for 5 minutes. After washing with 0.1% PBT they were blocked in 10% NGS for one hour and then incubated at 4^{o}C overnight with primary antibodies in 5% NGS and 0.1% PBT. Samples were then washed in 0.1% PBT for 2 hours and then incubated with secondary antibodies in 5% NGS and O.1% PBT. After 2 hours incubation at room temperature the samples were again washed for 2 hours in 0.1% PBT and then mounted on a slide with Vectashield. Primary antibodies used were as follows: Affinipure rabbit α-HRP (1:500, Jackson ImmunoReseach), guinea pig α-DVAP (1:1000, Pennetta *et al.,* 2002), Mouse α KDEL (1:50, Stressgen), mouse α HSP70 (1:200, Affinity BioReagents) Secondary antibodies were all from Jackson Immuno Research and were as follows: Goat α-rabbit FITC (1:100), goat α-GP Cy3 (1:500) and goat α-mouse cy3 (1:500)

2.8.3 Larval body wall staining

Wandering third instar larvae were selected and dissected in 1xPBS (phosphate buffered solution) and then fixed in Bouin's fixative (15:5:1 saturated picric acid, 37% formaldehyde and glacial acetic acid) for 5 minutes. The samples were washed in 0.1% PBT (PBS + 0.1% TritonX-100) and then blocked in 10% NGS in PBT for 1 hour under rotation. Samples were then incubated with the primary antibodies in 5% NGS in PBT at room temperature for 2 hours or at 4^{o}C overnight. After 2 hours of washing in PBT changing solution every 15 minutes the larvae were incubated for 2 hours with the secondary antibodies in 5% NGS in PBT and then washed for another 2 hours in PBT. Larvae were mounted on slides in VectaShield. The primary antibodies used were as

follows; rabbit polyclonal anti HRP (1:200, Jackson ImmunoResearch), mouse monoclonal anti KDEL (1:50, Stressgene), guinea pig polyclonal anti DVAP (1:1000, Pennetta *et al* 2002), mouse monoclonal anti HSP70 (1:200, Affinity Bioreagents), rabbit polyclonal anti-SERCA (1:1000, Sanyal *et al*, 2005), guinea pig polyclonal anti-Boca (1:1000, Culi and Mann, 2003) Co-staining with anti Boca required a different DVAP antibody so rat polyclonal anti-DVAP was used (1:1000, Pennetta *et al* 2002). Secondary antibodies were all used at a concentration of 1:500 (Jackson ImmunoResearch).

2.9 COS 7 cell transfections and immunohistochemistry

The open reading frame of DVAPT48I and wt DVAP were amplified from their pBluescript vectors by PCR using the following primers;

pCMV-Flag DVAP 5' (5'-CGAGTCGACCGTTCGTTTATGGCA) and pCMV-Flag DVAP 3' (5'-GCGGGAATTCGCCACAATGAGCAAATC)

pCMV-c-Myc T48I 5' (5'-CGCGGAATTCCCACAATGAGCAAATCA) and pCMV-myc 3' (5'-ATACGGTACCGGCGTTCGTTTATGGCA)

The amplified inserts were cloned into c-Myc- and Flag-tagged pCMV expression vectors. DCERT was also cloned into pCMV-c-Myc and Flag expression vectors by amplifying the ORF using the following primers:

pCMV DCERT Flag 5' (5'-CTGGCAGAATTCAAGAAGATG) and 3'(5'ATTAGGGCCCTCAGAACATGA)

pCMV DCERT c-Myc 5' CACTGGTACCTCAGAACATGATCGGCT) and 3' (5'-GCTAGAATTCGGAAGAGGATGGACACG)

COS-7 cells were cultured in DMEM medium containing 10% FCS and 1% penicillin/streptomycin. COS-7 cells were seeded on Poly-D-Lysine Coated glass coverslips (BD BioCoat) at a concentration of 150,000 cells/ml. The following day, cells

were transfected with Fugene 6 Transfection Reagent (Roche) according to manufacturer's instructions (see section 2.11). After 24 h the cells were fixed in 4% paraformaldehyde for 20 min and blocked in PBT containing 10% normal goat serum. Stainings with the relevant antibodies were performed accordingly to the procedure described above. The following primary antibodies were used: Rabbit anti-myc (Sigma) and mouse anti-Flag (Sigma) at a concentration of 1:500 and 1:200 respectively. Secondary antibodies (Jackson ImmunoResearch) were all used at a concentration of 1:500.

2.10 Scanning Electron Microscopy of the *Drosophila* eye

To observe the degenerative phenotype of the adult eye, *Drosophila* were decapitated under CO_2 and immediately fixed with 3% Glutaraldehyde in 0.1M Sodium Cacodylate Buffer (pH 7.4) for more than 3 hours. They were then washed in 0.1M Sodium Cacodylate buffer (pH7.4) for 1 hour changing every 20 minutes and incubated in 1% Osmium Tetroxide in 0.1M Sodium Cacodylate buffer for 1-2 hours. The samples were then washed in distilled water for 30 minutes, dehydrated in 50%, 70%, 90% acetone for 10 minutes each and then 3 times in 100% acetone and finally after drying with CO_2 in a Polaron E3000 SII CPD the samples are sputter coated with 20nm Gold/Palladium (60/40) in an EMSCOPE SC500 Sputter Coater. The samples were scanned with a Phillips 505 scanning eletron microscope (SEM). Fixing and preparation of the samples was carried out by Steve Mitchell of the EM facility at Edinburgh University and Dr Andrea Chai.

2.11 Co-immunoprecipitation

COS-7 cells were cultured in DMEM medium containing 10% FCS and 1% penicillin/streptomycin. 100,000-300,000 cells were plated in 2 ml of medium (50,000-150,000 cells/ml) in one well of a 6 well plate. The DNA for transfection was diluted to

a concentration of 0.5ug/ul meanwhile the Fugene 6 reagent (Roche) and serum free DMEM medium (Sigma) were warmed at room temperature. 6ul of Fugene was added to 94ul of DMEM in a sterile eppendorf, tapped to mix and then incubated for 5 minutes. 2ul of the diluted DNA was added to the reaction mixture, mixed and incubated for at least 15 minutes. The complex was then added dropwise to the cells, 100ul reaction per well and the cells incubated at 37^{o}C for 3 days. Cells were then trypsinized and resuspended in 1ml DMEM. The cells were lysed using Hepes-lysis buffer containing 1% Digitonin following the following protocol. A 2x stock solution of Hepes-Lysis buffer was made containing 100mM Hepes-NaOH buffer (pH7.4), 2mM EDTA and 100mM NaCl. To 5ml of this buffer the following were added; 5ml H_2O, 20µl DTT (0.5M), 100µl 100mM PMSF and 1 tablet protease inhibitor. Finally to 500µl, 5mg of Digitonin was added. The transfected cells were spun down at 2000 rpm for 5 minutes, washed by resuspending in 1 ml PBS, centrifuged again and resuspended in 160ul of the freshly prepared lysis buffer. The samples were incubated for 45 minutes on ice and after removing 10µl as input, the cell lysate was centrifuged at 13,000 rpm for 20min at 4^{o}C. The supernatant was incubated with 10ul of myc-beads (Pierce-c-myc IP/CO-IP kit) overnight at 4^{o}C in columns provided with the kit. The following morning the columns were centrifuged and the supernatant collected as it flowed from the column leaving only the beads and any interacting proteins behind. The column was washed 3 times in a 0.1% Digitonin wash buffer (as for lysis buffer but 10x less Digitonin) and then the myc tagged proteins eluted by incubating samples at 95^{o}C with 40ul non-reducing sample buffer. Samples from the beads, supernatant and initial input cell lysate were then run on a 12% SDS-PAGE gel for Western blot analysis.

2.12 Western Blot

The electrophoresis module (Biorad laboratories) was assembled and the protein samples and molecular weight marker (Molecular Probes) were loaded into each well of an 12% SDS-PAGE gel. Electrophoresis was carried out at 100 volts for 90 minutes.

The gels were removed and the protein transferred onto Hybond-P PVDF membranes (Amersham Biosciences) at 120V for 2 hours. The membranes were incubated in blocking solution which contains 2% ECL Advance Blocking Agent (Amersham Biosciences) diluted in TBST (Tris buffered solution with Tween)-0.1% Tween 20 (Sigma) in TBS (8 grams NaCl, 20ml 1M Tris at pH 7.6 brought up to 1 litre volume with distilled water). The following morning, the membranes were rinsed briefly in TBST and then incubated with primary antibodies in blocking solution for 2 hours with gentle agitation. Membranes were then washed vigorously for 2 hours in TBST changing solution every 15 minutes. The membranes were then incubated for two hours with the secondary antibodies with gentle agitation. Following this final incubation the membranes were again washed for 2 hours in TBST. Signals were detected on the membranes using the ECL Advance Western Blotting Detection Kit (Amersham Biosciences). Soutions A and B in the kit were mixed at a 1:1 ratio and then pipetted onto membrane in a thin layer and left for 1 minute. Excess was removed by blotting on a paper towel and then the membrane wrapped in cling film. In a dark room the membranes were placed in an X ray film cassette and exposed to autoradiography film (Hyperfilm^{TM}ECL) for 60 seconds. The film was then submerged in developer for 30-60 seconds, rinsed in water and then submerged in fixer.

The antibodies used for the Western were as follows. Primary antibodies: Guinea pig anti DVAP (GP33, 1:40,000) and rabbit anti c-myc (Sigma, 1:10,000) Secondary antibodies were all from Jackson ImmunoResearch and were as follows. Goat anti rabbit HRP (1:30,000) and goat anti guinea pig HRP (1:60,000)

2.13 The yeast two-hybrid System

We used the MATCHMAKER GAL4 Two-Hybrid System 3 for our yeast two-hybrid and the *Drosophila* wild-type VAP as the bait protein to screen a *Drosophila* embryonic library provided by Clontech. This system incorporates a number of features that reduce the incidence of false positive results. The yeast strain used is AH109, (*MATa, trp1-901,*

leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3 : : MEL1UAS-MEL1 TATA-lacZ)(Clontech) which virtually eliminates false positives through the incorporation of three reporters; *ADE2, HIS3*, and *MEL1* (or *LacZ*), all under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes. These promoters yield strong and specific responses to GAL4 which means that false positives caused by proteins interacting directly with the sequences flanking the GAL4 binding site and those that interact with transcription factors bound to specific TATA boxes should not occur. The *ADE2* reporter allows yeast to grow on medium lacking Adenine and this alone provides strong nutritional selection. Using *HIS3* selection (media lacking in Histidine) reduces the incidence of false positives and increases the stringency of selection. There is also the option of using either *MEL1* or *LacZ,* which encode α-galactosidase and β-galactosidase, respectively. In this screen we are using *MEL1* because α-galactosidase is a secreted enzyme so it can easily be assayed directly on plates containing X-α-Gal. In the occurrence of an interaction, not only will the yeast be able to grow on plates lacking Adenine and Histidine but the colonies will be blue in colour due to the secretion of α-galactosidase. Provided in this kit are two types of cloning vectors pGBKT7 contains the GAL4 binding domain and pGADT7 contains the activation domain. Full length and truncated forms of DVAP bait constructs were generated by cloning cDNA fragments in frame alongside the Gal4 binding domain coding sequence in pGBKT7 vector. The oligonucleotides listed in Table 2.4 were used to amplify the various fragments from the full length cDNA clone LD06780 for *DVAP* and GH08349 for *SAC1* (received from the DGRC) by PCR using *Pfu* turbo polymerase (Stratagene). The same oligonucleotides could be used to clone the ORF of these genes into pGADT7 in frame downstream of the GAL4 activation domain coding sequence to produce a target fusion protein.

2.13.1 The Drosophila embryonic library

A *Drosophila* 0-24 hour embryonic library was obtained from Clontech. This is a library of cDNA inserted into pACT2 vectors. Like pGADT7, this vector contains a GAL4 activation domain and will initiate transcription when brought into close proximity to a bait protein containing the GAL4 binding domain. During construction of the library cDNA is initially inserted into the lambda phage ACT2 and then released into *E.coli*. The average size of the cDNA inserted into the pACT2 vectors is 1.4kb and the estimated number of independent colonies is 3 x 10^6.

Primer Name	Restriction site	Primer Sequence 5'-3'
DVAP 5'	EcoR1	GCCAGCGAATTCGCCACAATGAGCAAATCA
DVAP 3'	BamH1	TAGTGTGGATCCTAGCATGTTGCTGGCGTT
C-term DVAP	EcoR1	GCAGGAATTCGCAAATGCTGAGAACACC
N-term DVAP	BamH1	GAATGGATCCGGTGTTCTCAGCATTTGC
Sac1 5'	Nde1	TGCACATATGATCATGGACAGCAGGGAG
Sac1 3'	BamH1	TTACGGATCCACGACACTAACGTCATGG
C term Sac1	Nde1	GCAGCATATGGAGTCTTTCGACTTCCAC
N-term Sac1	BamH1	GATTGGATCCGATGAGTATGTTCAGCCG
Short Sac1 3'	Kpn1	GAGCGGTACCTACATTGCAGTTAATCG
hVAP 5'	EcoR1	TTGAGTGAATTCATGGCGAAGGTGGAGCAG
hVAP 3'	BamH1	GTAGAAGGATCCATGCTACCTCTACAAGGC
Pio 5'	EcoR1	TGCCGAATTCACCATGAAGACAGGCACT
Pio 3'	BamH1	TGTAGGATCCTCCATCCTCCTCATCTTC
Pio–N term	EcoR1	CAGCGAATTCCAGATTCCAACGGCAATG
Pio TM domain	EcoR1	CATAGAATTCGACAGCAAGGAGGAGGAC

Table 2.4 Primers from the yeast two hybrid

2.13.2 Titration and amplification of the Library

The library of cDNA clones was obtained from Clontech and, upon arrival, the first step was to titer the library. Various dilutions of a library aliquot were plated on LB plates containing Ampicillin and incubated at 30^{o}C for 24 hours. The titer (cfu/ml) was then calculated by counting the number of colonies.

(Initial dilution ½) Dilution: 10^{-6} No. of colonies: 177 Amount plated: 100µl

177 x 1/100 x 10^6 = 1.77 x 10^6 = 1.77 x 10^9 colonies per ml. x 2 = **3.2 x 10^9** cfu (colony forming units)

It is suggested that 2-3 times no. of independent colonies should be plated = 9 x 10^6 at a concentration such that the colonies are almost confluent on the 150mm plates ~ 20,000 9,000,000/20,000 = 450 plates.

The plates were incubated at 30^{o}C to avoid any plaques due to phage contamination as a by-product of the library construction in the lambda phage. Plates were incubated for 36 hours. To remove the colonies, 5 ml of LB media was pipetted onto each plate and then the bacteria scraped into the solution using a bent Pasteur pipette and then removed and pooled together. The resulting 2.25 litres of bacteria culture was then left shaking at 30^{o}C for 3 hours to help the bacteria recover and then sterile glycerol added to a final concentration of 25% and the solution aliquoted and put at -80^{o}C. A third of the culture was set aside for plasmid preparation and this was divided between 6 Beckman centrifuge flasks and pelleted down by centrifuging at 6000rpm for storage at –20^{o}C. The plasmids were purified using a QIAGEN Plasmid Giga kit that follows a protocol based on a modified alkaline lysis procedure followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin in a column.

2.13.3 AH109 Stock

The AH109 yeast strain is provided in the kit as a frozen glycerol stock. This must first be streaked onto YPDA plates before a colony picked and the yeast plated onto selective SD plates missing the vital nutrients Methionine and Uracil. The yeast colonies are allowed to grow for 1-2 weeks until the colonies are about 2mm in size and then the plates sealed and stored in a fridge for a maximum of 2 months before they should be replated. For use to inoculate a liquid culture the yeast colonies should be about 1 week old and ideally incubated at a constant temperature of 30^{o}C and if the colonies are less than 2mm in size, a number of colonies should be picked.

2.13.4 Transfection protocol

Initially 50ml of YPDA is inoculated and incubated overnight for 16-18 hours at 30^{o}C until the OD_{600} is greater than 1.5. The culture is then diluted into a culture of 300ml YPDA so that the OD_{600} is 0.2-0.3 and again incubated at 30^{o}C for at least 3 hours until the OD_{600} is 0.5±0.1. The yeast culture is then pelleted down in sterile falcon tubes using a tabletop centrifuge at 1000 x g for 5 minutes. The pellets are resuspended in sterile ddH_2O and combined into a total volume of 20-50 ml. Recentrifuge, remove supernatant and resuspend in a total volume of 1.5ml 1xTE/LiAC. The cells are now competent and ready for transfection. Herring testes carrier DNA provided by clontech is mixed with the vector DNA to be transfected and the cells added along with a polyethylene glycol/LiAC/TE solution. PEG is necessary for inducing the binding of DNA to the yeast cell and Li^{+} permeabilises the cell wall allowing the DNA to enter and bind to the cell membrane. Finally with the addition of Dimethyl sulfoxide (DMSO) and a 15 minute heat shock the integrity of the plasma membrane is damaged and it is permeabilised for the entry of vector DNA. The preps are put on ice for two minutes after the heat shock and then pelleted down briefly and resuspended in 500ul TE pH 7.5 or YPDA. 200µl is plated onto the selective SD Drop out plates, -Leu,Trp to select for the presence of both vectors and -Adenine, Histidine, Leucine, Tryptophan (AHLT) for an interaction.

2.13.5 Library Scale Yeast two Hybrid Screen

A *Drosophila* 0-24 hour embryonic library containing a library of cDNA inserted into the pACT2 vector containing the GAL4 activation domain was obtained from Clonetech. Following amplification and titering of the library as described above yeast strain AH109 was cotransfected with the full length DVAP BAIT plasmid and library plasmids following the protocol outlined previously but with an initial culture of 150ml

diluted into 1 litre. A final volume of 10ml transfected yeast is obtained which is plated on 50 large 150mm AHLT plates.

2. 14 Reagent Stocks

EDTA (0.5M) pH 8. Add 186.1g disodium EDTA.2H_2O to 800ml of H_2O, adjust the pH to 8.0 with NaOH and bring up the volume to 1 litre

SOB Per liter: 20g Tryptone, 5g Yeast extract, 0.5g NaCl. Add dd H2O to a final volume of 1liter and autoclave. Then add 10ml filter sterilized 1M MgCl2 and 10ml filter sterilized 1M MgSO4 prior to use. Store at –20^{o}C or –80^{o}C in 50ml aliquots

SOC Per 100ml: Add 2ml filter sterilized 20% glucose or 1ml filter sterilized 2M glucose. Bring up to 100ml with SOB (autoclaved)

Sodium Acetate (3M, pH5.3): Dissolve 408.3g of Sodium Acetate.3H_20 in 800ml of H_2O. Adjust pH to 5.3 with glacial acetic acid and adjust volume to 1 liter with H_2O.

STET: 10mM Tris-Cl pH 8.0, 0.1M NaCl, 1 mM EDTA pH 8.0 and 5% (v/v) Triton X-100

Tris-Cl (1M): Dissolve 121.1g of Tris base in 800ml of H2O. Adjust pH to desired value by adding concentrated HCl. For pH 8.0 add 42ml HCl. Bring up the volume to 1 litre. Sterilize by autoclaving

10 x Tris EDTA (TE) pH 8: 100mM Tris-Cl (pH8) and 10mM EDTA (pH8)

50x TAE: 242g Tris base**,** 57.1ml glacial acetic acid**,** 100ml of 0.5M EDTA (pH8.0) Bring up to 1 liter with ddH2O

Antibiotic Concentrations

	Stock concentration	Working Conc.	In 100ml
Ampicillin	100mg/ml	100ug/ml	100ul
Kanamycin	10mg/ml	50ug/ml	500ul
Chloramphenicol	34mg/ml	68ug/ml	200ul

Chapter 3: Results

3.1 Characterization of the T46I mutation using *Drosophila*

As described earlier, there has recently been a discovery by the lab of Prof. Jaqueline de Belloroche of Imperial College London of a new mutation in VAPB (T46I) that is also believed to be causative for ALS. Such a discovery adds weight to the role played by VAPB in ALS as despite there being a large number of VAPP56S affected individuals, the fact that they are all related and carry the same mutation still provides room for argument against the worth of this mutant protein in ALS research. The T46I mutation has been identified in a completely unrelated family and is also within the MSP domain of VAP which is highly conserved from yeast to man. Two mutations linked to ALS and both affecting residues of such a conserved domain make the case much stronger for the necessity of research into VAP proteins with respect to ALS and perhaps other degenerative diseases. It is now necessary to characterize the VAPT46I mutant protein and *Drosophila* is the ideal model system for this.

The mutation in humans was mapped to the corresponding residues in *Drosophila* by aligning the primary sequences for these two proteins. The corresponding Threonine mutated in *Drosophila* is at position 48 (T48I) and site directed mutagenesis on the wt cDNA clone of DVAP (LD06878) was carried out to generate mutated cDNA which was then subcloned into a *Drosophila* transformation vector (UAS-DVAPT48I). The construct was injected into *Drosophila* embryos and the P element transposase recognition sites within the pUAST vector, enables the insertion of DNA into the *Drosophila* genome when transposase is present. A number of transgenic lines were established and the transgene expressed using the GAL4 UAS system as described in Chapter 1.4 (Brand and Perrimon 1993). A number of experiments have been carried out to observe the effect of this mutation on DVAP *in vivo* as compared to the wild type and original P58S mutation.

3.2 Alignment of amino acid sequence for DVAP and hVAPB

A

```
HVAPB 1   MAK--VEQVLSLEPQHELKFRGPFTDVVTTNLKLGNPTDRNVCFKVKTTA     48
          |:|  .:..|::||:|||:|.||||..|.|.:.|.|.:...:.||:||||
DVAP  1   MSKSLFDLPLTIEPEHELRFVGPFTRPVVTIMTLRNNSALPLVFKIKTTA     50

      49  PRRYCVRPNSGIIDAGASINVSVMLQPFDYDPNEKSKHKFMVQSMFAP--     96
          |:||||||.|.|....|..|.:.||||.||..||:|||||||:.||
      51  PKRYCVRPNIGKIIPFRSTQVEICLQPFVYDQQEKNKHKFMVQSVLAPMD    100

      97  TDTSDMEAVWKEAKPEDLMDSKLRCVFELPAENDKPHDVEINKII-----    141
          .|.||:..:||:.:||.|||:||:||||:|.......:......:
     101  ADLSDLNKLWKDLEPEQLMDAKLKCVFEMPTAEANAENTSGGGAVGGGTG    150

     142  --------STTASKTETPIVSKSLSSSLDDTEVKKVMEECKR---LQGEV    180
                  :.|:|.:...:.||...||.|..:...::|..:.   |.|||:
     151  AAGGGSAGANTSSASAEALESKPKLSSEDKFKPSNLLETSESLDLLSGEI    200

     181  QRLREENKQFKEEDGLRMRKTV----------QSNSPISALAPTGKEEGL    220
          :.|||.|.:.:.|: |.::..:          |.|.|   .||...|:.:
     201  KALRECNIELRREN-LHLKDQITRFRSSPAVKQVNEP---YAPVLAEKQI    246

     221  STRLLALVVLFFIVGVIIGKIAL    243
          ....:|:.:...||.:::||..|
     247  PVFYIAVAIAAAIVSLLLGKFFL    269
```

B

Yeast	FKVR**T**SAPTKYCVR**P**N
Aplysia	FKVK**T**TAPKRYCVR**P**N
Drosophila	FKIK**T**TAPKRYCVR**P**N
Mouse	FKVK**T**TVPRRYCVR**P**N
Human	FKVK**T**TAPRRYCVR**P**N

Figure 3.1 Comparison of VAP sequences
A) Alignment of the human and *Drosophila* VAP protein sequence. Lines joining two letters indicate complete conservation, dots indicate moderate conservation (: more so than a single dot). The MSP domain is boxed and spans 100 amino acids in *Drosophila*, 96 in humans. The two mutated residues that have been linked to ALS are the P56S and T46I in humans shown by the green highlight. These correspond to P58S and T48I in *Drosophila*. They both fall within the 16 amino acid sequence (highlighted in pink) which is highly conserved from yeast to man as shown in panel B. The yellow region is the coiled coil domain and the blue region is the transmembrane domain. Figure is adapted from Chai *et al* 2008. Sequences are from Flybase and NCBI GenBank and alignment was carried out using the online Emboss Pairwise Alignment tool.

3.3 Neuronal over-expression of DVAPT48I in *Drosophila* larvae induces aggregate formation

Previous work looked at the effect of over-expressing *DVAPP58S* in *Drosophila* larvae under the control of the pan neural *elav-gal4* driver and found that the mutant protein formed large aggregates in the nerve fibers and cell bodies of the larva brain when expressed together with the endogenous wt protein (Chai *et al.* 2008). In wt larvae the localisation of DVAP is homogeneous along the length of the nerve fiber and in the membrane and cytoplasm of the cell bodies but this is lost and replaced by bright punctate staining upon transgenic expression of the pathogenic mutant protein. This suggests that the presence of DVAPP56S leads to the formation of aggregates and sequesters DVAP away from its normal localisation. See figure 3.2.

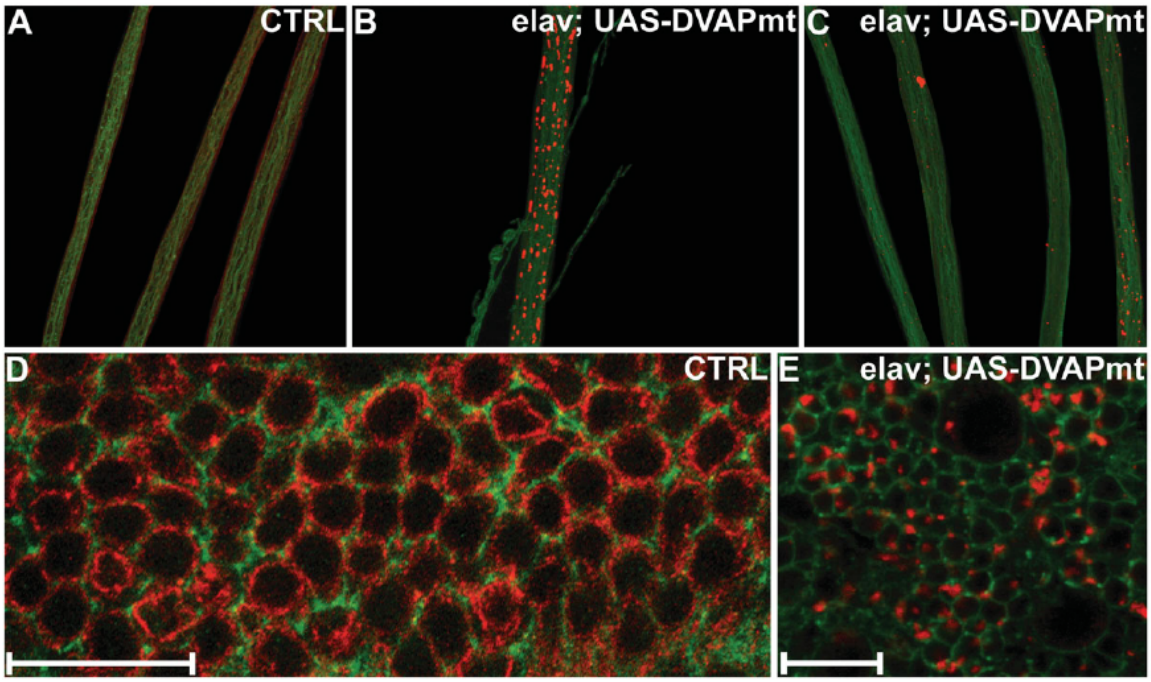


Figure 3.2 Transgenic DVAPP58S expression induces aggregate formation in the larval brain and nerve fibers

Nerve fibers and brains of third instar larvae stained with antibodies for DVAP (red) and the cell surface marker anti-HRP (green). A) Wt (Canton S) larvae show faint but uniform DVAP staining along the length of the nerve fiber. B) Larvae expressing transgenic *DVAPP58S* under the control of the elav-GAL4 pan neural driver present with concentrated spots highly immunoreactive for DVAP marking the presence of aggregates along the nerve fiber in regions just prior to the brain or the muscle endplate but not in between these regions (C). D) Brains of wt larvae show uniform staining of the plasma membrane around the cell bodies and similarly to the nerve fibers this is reduced to punctate staining in the brains of larvae expressing transgenic *DVAPP58S* (E). In (D) and (E), single sections of confocal images are shown. Scale bars are 20 um. Images taken from Chai *et al* 2008.

Following the creation of the *DVAPT48I* transgene it is important to see if it too induces the formation of aggregates in the *Drosophila* nervous system. Similarly to the previous experiment (Chai *et al.* 2008), *DVAPT48I* was expressed under the control of *elav-GAL4* and the localisation of DVAP examined in the larva nerve fibers and cell bodies using antibodies specific for DVAP (GP33) and the neuronal cell surface marker (HRP). Wt (Canton S) and transgenic third instar larvae were dissected and stained with the two antibodies and a confocal analysis of the brain and nerve fibers carried out (See figure 3.3).

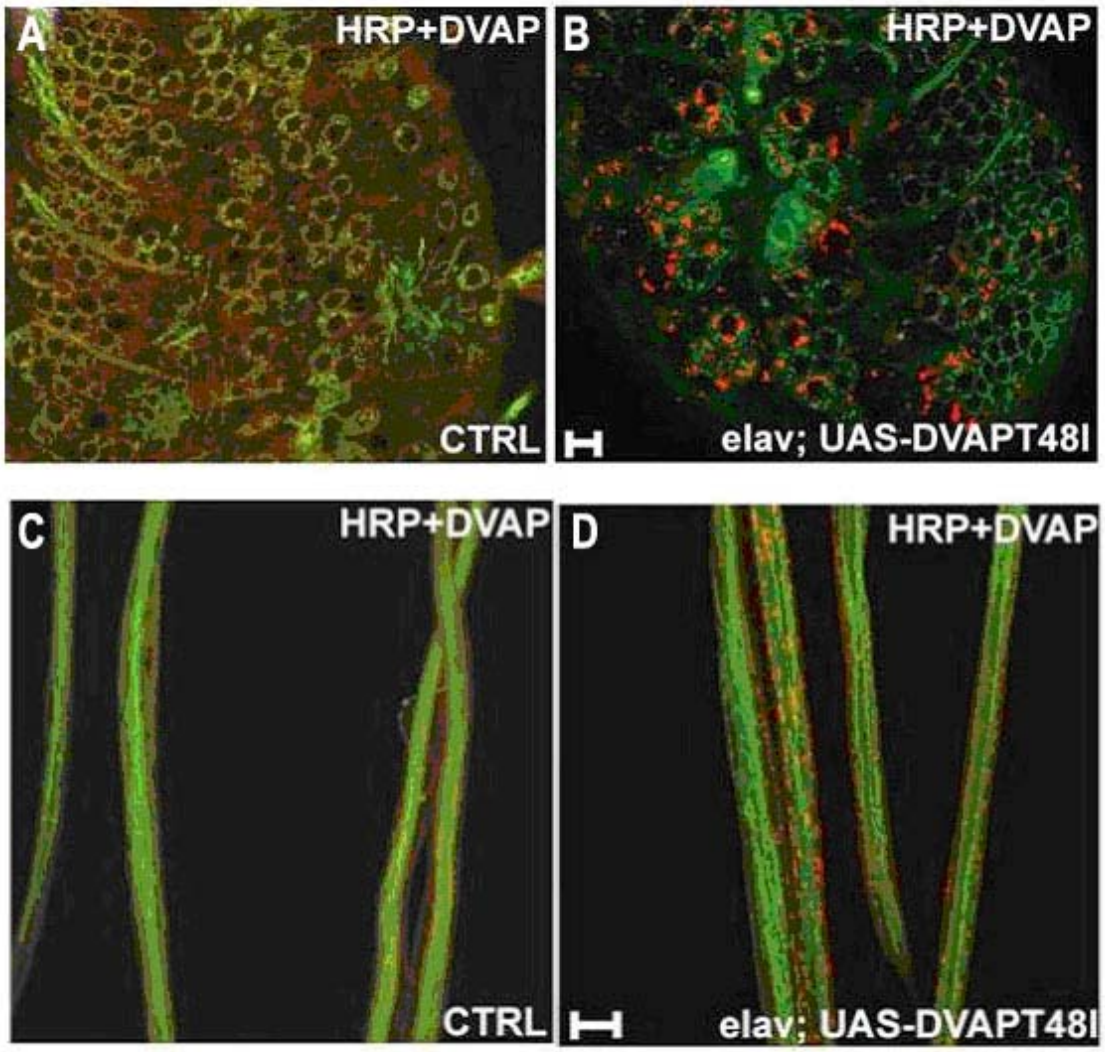


Figure 3.3 Transgenic DVAPT48I expression induces aggregate formation in the larval brain and nerve fibers

Brains and nerve fibers of third instar larvae were stained with antibodies against DVAP (red) and the neuronal cell surface marker anti-HRP (green). A) Control wt larvae (Canton S) have uniform staining for DVAP around the cell bodies of the nerve. B) In *DVAPT48I* larval brains DVAP staining is reduced to bright punctuate, suggestive of aggregates. C) Nerve fibers from wt larvae present with a faint and uniform staining for DVAP while in T48I transgenic nerves, large aggregates that are strongly immunoreactive for DVAP accumulate along the nerves (D). Scale bars are 10μm.

The results from the transgenic larvae expressing *DVAPT48I* in a wt background showed that in the presence of the endogenous protein, aggregates formed that are brightly immunoreactive for DVAP and there is a reduction in staining for DVAP in other areas of the nerve (figure 3.3). The data are identical to those seen with the previous mutation,

P58S (figure 3.2), and are suggestive of a similar disease mechanism. If the hypothesis is correct that the formation of aggregates sequesters wt DVAP away from its normal localisation then the composition of the inclusions would be of both mutant and wt protein. In the previous experiments this could not be proven as the DVAP antibody detected both forms of the protein. To further analyse the aggregates, COS7 cells were utilized to examine the composition of such inclusions following the expression of tagged wt and mutant DVAP proteins.

3.4 Composition of aggregates following expression of DVAPT48I

In order to differentiate between the mutant and wild type DVAP, Flag- and c-Myc-tagged fusion proteins were created by subcloning the cDNA into pCMV vectors containing the tag sequence upstream of the multiple cloning site. The resulting N-terminally tagged *DVAP-Flag* and *DVAPT48I-Myc* were expressed in pCMV under the control of the cytomegalovirus promoter in COS7 cells following the co-transformation of the two plasmids into this cell line. Anti-c-Myc and anti-Flag staining allowed for the individual localisation of the two fusion proteins when both are expressed together.

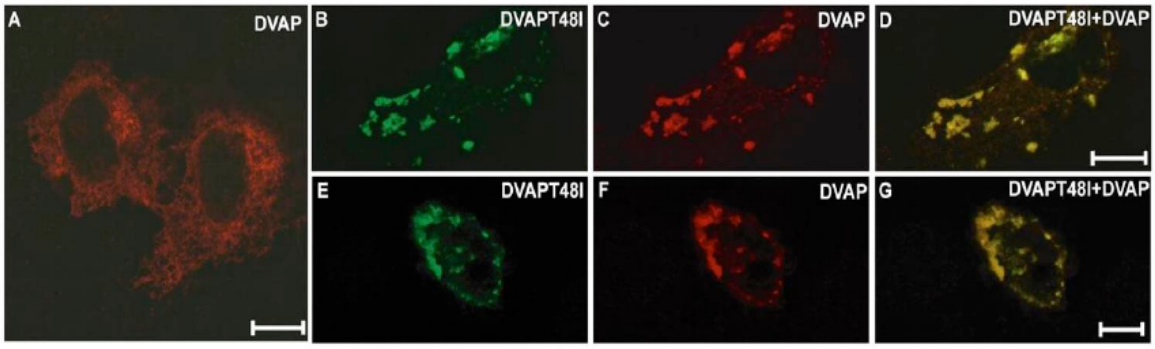


Figure 3.4 Cotransfection of COS7 cells with DVAP and DVAPT48I induces aggregate formation composed of both wt and mutant protein
COS7 cells transfected with *DVAP-Flag* and *DVAPT48I-c-Myc* under the control of the cytomegalovirus promoter and stained with antibodies specific for the two tags. A) Staining against Flag in control cells expressing *DVAP-Flag* alone show a uniform staining throughout the cytoplasm in a reticulum pattern that is consistent with an ER-associated protein. B-G) Cells co-transfected with wt and mutant DVAP show a staining pattern suggesttive of aggregate formation. D and G) Anti-Flag and Anti-c-Myc staining show colocalisation suggesting that the aggregates are composed of both wt and mutant DVAP.

Anti-flag staining of the COS-7 cells transfected with DVAP alone is of a uniform perinuclear reticular staining pattern as would be expected for an ER-associated protein (figure 3.4). However when the COS-7 cells are cotransfected with both wt and mutant protein the staining becomes more diffuse with punctuate staining highly immunoreactive for both Flag and c-Myc tags. This staining is very similar to that seen in the cell bodies of *Drosophila* larvae and is suggestive of the formation of aggregates in the cells. The colocalisation of the staining shows that the aggregates are indeed composed of both wt and mutant protein.

3.5 DVAPT48I induces aggregate formation and sequesters wt DVAP away from its normal localisation

The experiments described here have clearly shown that the T48I mutation in DVAP brings about a similar phenotype at a cellular level to that seen when *DVAPP58S* is expressed in the *Drosophila* larval nervous system (Chai *et al.* 2008). In the nervous

system DVAP is faintly but uniformly expressed along the nerves and at the synapse. Following the addition of mutant transgene (T48I or P58S), DVAP accumulates into aggregates which mainly concentrate at the proximal and distal ends of the nerve. A lack of staining between these points suggests a sequestering of DVAP away from these areas of the nerve. Previously we did not answer the question of whether the *DVAPP58S* induced aggregates contained both wt and mutant protein but now, using cell culture, we have shown for *DVAPT48I* that the inclusions, which only occur when both proteins are present, do indeed contain both wt and mutant DVAP. Following the work presented on the *Drosophila* model of ALS which used *DVAPP58S* to induce many major hallmarks of ALS including the neuronal inclusions as well as muscle wasting, neuronal degeneration and locomotion defects, a number of groups looked at the possible mode of action for the phenotypes witnessed. We put forward a dominant negative action in the belief that the mutant protein would interfere with the localisation of the wt protein. This is despite it having the ability to rescue the null phenotype and retaining a number of its essential functions. This is perhaps due to an excessive wt function so the mutant protein is in fact a hypermorphic allele which causes it to bind strongly to wt DVAP and induce aggregation. This then inhibits the action of the wt protein and therefore the final outcome is that of a dominant negative mechanism. Following our initial model, other groups supported this hypothesis and have shown in both cultured motor neurons and in *Drosophila* larva muscles that wt DVAP is brought into aggregates when *DVAPP58S* is expressed (Ratnaparkhi *et al.* 2008; Tsuda *et al.* 2008). Using COS7 cells and GST pull-down Teuling *et al* have also shown that endogenous hVAPB is brought into insoluble aggregates in the presence of hVAPP56S (Teuling *et al.* 2007). The results that we report here are suggestive of a similar story for DVAP in the presence of the mutant protein DVAPT48I. If this is indeed true, it may well be a similar case for VAPBT46I in humans.

3.6 Effect of *DVAPT48I* expression on the ER and chaperone HSP70

VAP proteins are type II integral membrane proteins and have been shown to localise to the ER in yeast and mammals (Kagiwada *et al.* 1998; Soussan *et al.* 1999). The wt staining of DVAP has a pattern that is conducive to an ER localised protein and the question to be answered now is whether, following the expression of DVAPT48I, are the resulting cytoplamic aggregates linked to a change in the structure of the ER? Teuling *et al* describe the formation of aggregates in *DVAPP56S* expressing HeLa cells which they reported to contain tubular structures of ER origin (Teuling *et al.* 2007). Co-expression of VAP along with Nir2 has also been shown to affect the structural integrity of the ER in HeLa cells (Amarilio *et al.* 2005) and removal of CERT, another interactor of VAP proteins, causes ER stress as seen by engorgement of this organelle (Wang *et al.* 2009b). DVAP acts as the ER anchor for both these FFAT containing proteins and so, following the aggregation and sequestering of wt DVAP it may be expected that there will be a direct effect on the structure of the ER. To investigate the cytoplasmic localisation of DVAP in *Drosophila*, larval brains were stained with antibodies against DVAP and the ER marker KDEL. The ER was also observed following the expression of *DVAPT48I* transgene.

Along with changes in the ER, it would be expected that in conjunction with the formation of aggregates there will be a change in the pattern and level of heat shock proteins in the cell. Under wt conditions, these chaperones normally prevent the accumulation of misfolded proteins by aiding their refolding or facilitating degradation by the proteasome (Shinder *et al.* 2001). Chaperones are upregulated in response to cellular stress and in the $SOD1^{G93A}$ model of ALS, an upregulation of HSP70 has been observed (Liu *et al.* 2005). The benefit of such a response is still under debate as unlike the results seen in primary culture and other degenerative disease, an increase in endogenous HSP70 did not extend the lifespan of the SOD1 mice and HSP70 was actually reported to be sequestered into the SOD1 aggregates (Shinder *et al.* 2001; Liu *et al.* 2005). However in another study the addition of exogenous recombinant HSP70 was

found to extend the lifespan and delay disease onset in the SOD1^{G93A} mouse (Gifondorwa *et al.* 2007). Some reports suggest that motor neurons have an intrinsic inability to respond to cellular stress and have a higher threshold for the induction of the heat shock protein response making these neurons more vulnerable to cellular stress (Batulan *et al.* 2003) The involvement of HSP70 in our *Drosophila* model of ALS is obviously very interesting and as such we stained brains of *DVAPT48I* transgenic larvae with an HSP70 antibody and carried out confocal analysis (figure 3.5).

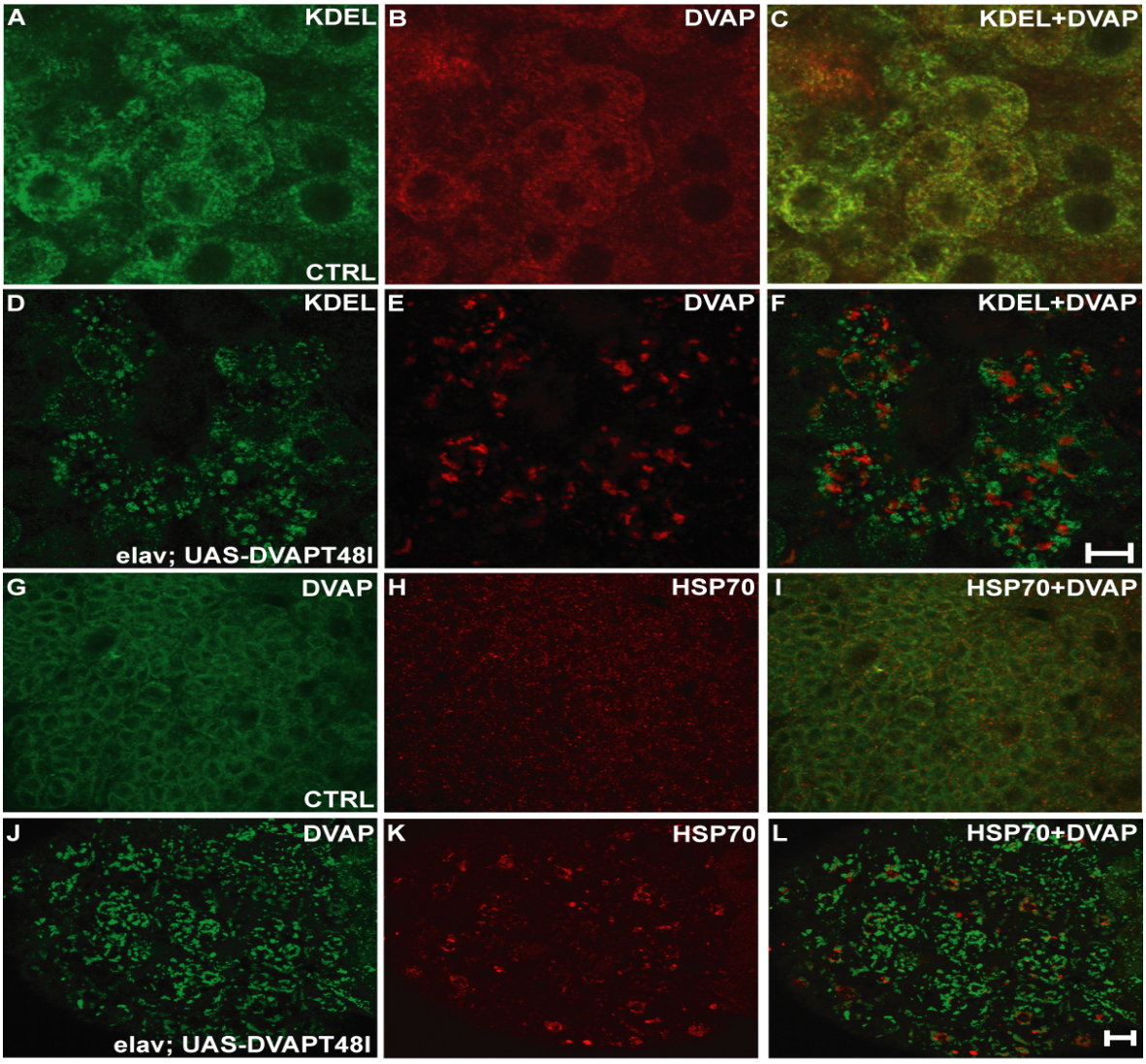


Figure 3.5 Expression of DVAPT48I in the larval brain is associated with fragmentation of the ER and an upregulation of HSP70
Brains from wt (Canton S) larvae (A-C, G-I) and transgenic lines expressing DVAPT48I (D-F, J-L). A-F) Brains are stained with antibodies specific for DVAP (red) and the ER marker KDEL (green). Fragmentation of the ER accompanied by DVAP aggregates is seen in the brains of transgenic larvae. G-L) Brains are stained with antibodies specific for DVAP (green) and HSP70 (red). In wt neuronal cell bodies, HSP70 staining is faint but uniform. In transgenic larvae, there is an up-regulation of HSP 70 and some aggregation is seen close to but not overlapping with those of DVAP. Scale bars are 10 μm.

Results from the co-staining of *Drosophila* larval brains with antibodies against DVAP and KDEL are suggestive of a good colocalisation of DVAP with the ER in the cytoplasm of the brains. In transgenic flies expressing the pathogenic mutation, DVAP forms aggregates and the ER staining is nolonger uniform with some areas of colocalisation within the inclusions. A possible explanation is that the structure of the ER has been altered and become fragmented although it may also be linked to a redistribution of the KDEL marker. For this reason the phenotype was also confirmed using an antibody specific for the Boca protein (data no shown), another independent ER marker (Culi and Mann 2003). Other ER markers that could have been used are Calnexin and Protein Disulphide Isomerase (PDI). The change in staining for ER and its apparent fragmentation should be examined further at an ultrastructural level by TEM. Whether this is a direct result of the removal of DVAP or due to an indirect effect on the change in localisation of interacting proteins such as Nir2 and CERT is something to be considered. If the removal of DVAP into aggregates is causing ER stress, perhaps by an increase in the level of Ceramide in this organelle, this opens up a possible pathway that might lead to the death and degeneration of the cell. This will be elaborated upon in the Discussion.

The results from the staining observed for HSP70 in wt larva brains is uniform throughout the cytoplasm of the cell bodies. (figure 3.5 G-I). The staining in the brains of *DVAPT48I* transgenic larvae is indicative of an upregulation of HSP70 with the formation of some aggregates that are highly immunoreactive for HSP70. Although these aggregates are closely associated with the DVAP inclusions present in these cells, they do not overlap.

The results reported here are possibly suggesting that following the pan neural transgenic expression of *DVAPT48I*, the neuronal cells become stressed as seen by the accumulation of aggregates, the upregulation of heat shock protein 70 and the alteration of the structure of the ER. This is highly suggestive of the pathomechanism of the disease in VAP-linked ALS and perhaps other forms of ALS and degenerative disease. The next step is to look at the effect of the transgene when expression is localised to the muscles as muscle wastage is a key hallmark of ALS and it is unknown whether this is a direct effect of the mutant protein or due to the loss of nerve input following neuronal degeneration.

3.7 Effect of *DVAPT48I* expression when targeted in the muscle

One of the early signs of ALS is muscle weakness and atrophy of the skeletal muscles. However, as the understanding of ALS has increased it is becoming increasingly obvious that ALS is not a disease solely affecting the motor neurons. It is important to consider whether the observed atrophy at the muscle is secondary to a loss of nervous input or indeed as a direct result of toxicity within the muscle. A study using the mutant SOD1 mouse model of ALS found that localised expression of $SOD1^{G93A}$ in the muscle causes muscle atrophy in mice as young as 4 weeks old. Ultrastructural analysis showed misalignment of sarcomeres, disorganization of the sarcotubular system and a disruption of the mitochondrial morphology (Dobrowolny *et al.* 2008). A few other studies have also used the SOD1 mice to show that muscle targeted variation in gene expression can affect disease progression. Muscle localised expression of insulin like growth factor (Igf) -1 delayed the progression of disease (Dobrowolny *et al.* 2005) and Nogo-A, a neurite outgrowth inhibitor shows increased expression in the skeletal muscle of SOD1 mice and ablation increased the lifespan of the mice whereas overexpression in the muscle fibers caused a reduction in the NMJ size (Dupuis and Loeffler 2009).

To examine whether mutant VAP is directly toxic to the muscle fibers in *Drosophila,* expression of the *DVAPT48I* transgene was driven in the muscles using a

BG57-GAL4 line in which GAL4 expression is under the control of a muscle-specific enhancer (Budnik *et al.* 1996). Both wt and *BG57-GAL4; UAS-DVAPT48I* lines were dissected and the NMJs stained with antibodies specific for DVAP and a range of muscle specific markers in order to assess the sub-cellular localisation of DVAP and the effect that mutant VAP might exert on the muscle. Similar to the larval brains, the effect of *DVAPT48I* expression on HSP70 was also examined at the larval NMJ using antibodies specific for this endogenous chaperone.

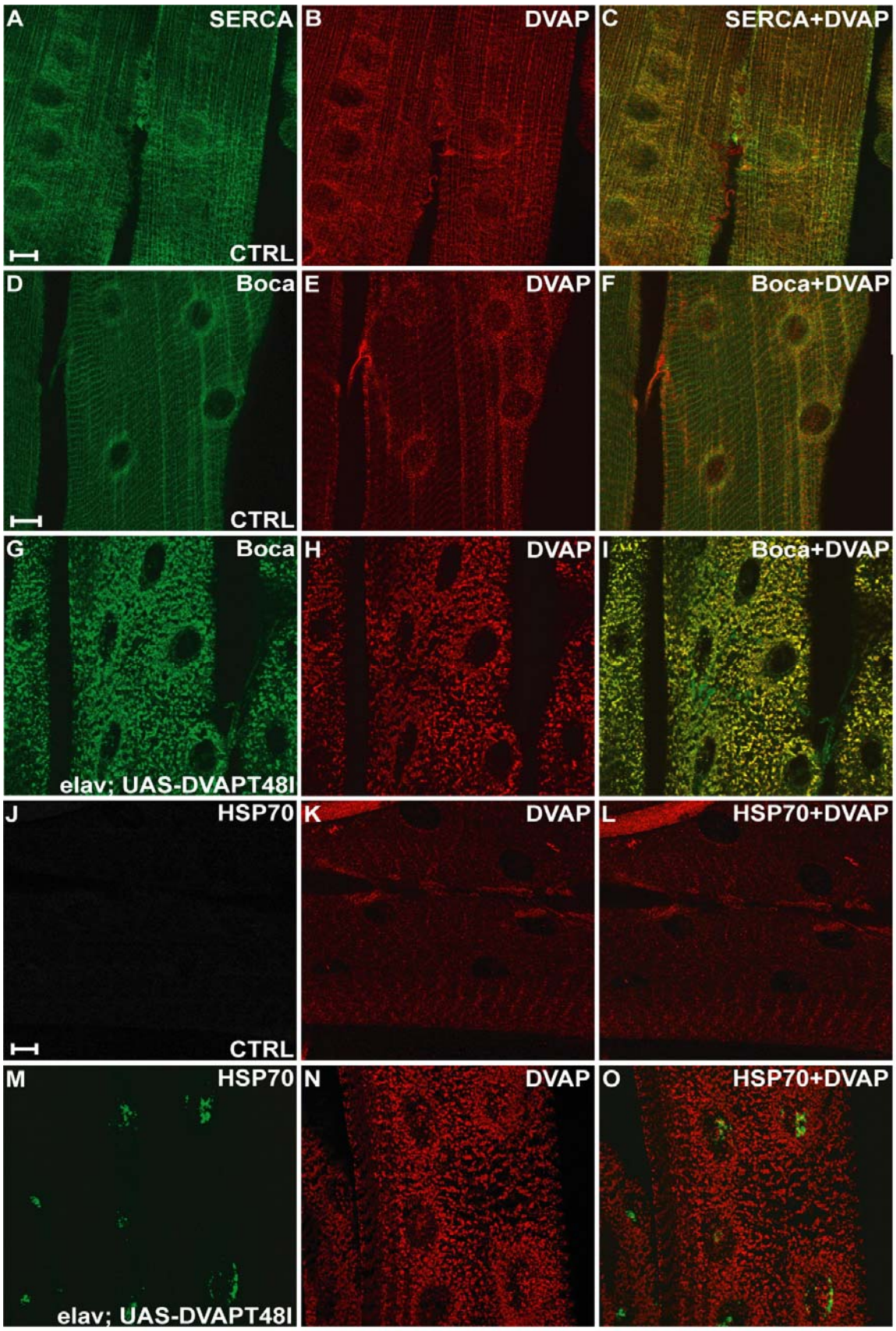

A
SERCA
CTRL
B
DVAP
C
SERCA+DVAP
D
Boca
CTRL
E
DVAP
F
Boca+DVAP
G
Boca
elav; UAS-DVAPT48I
H
DVAP
I
Boca+DVAP
J
HSP70
CTRL
K
DVAP
L
HSP70+DVAP
M
HSP70
elav; UAS-DVAPT48I
N
DVAP
O
HSP70+DVAP

Figure 3.6 Targeted DVAPT48I expression in the muscles induces aggregate formation, ER fragmentation and an upregulation of HSP70
Larval muscles from wt (Canton S) larvae (A-F, J-L) and transgenic larvae expressing UAS-DVAPT48I in the muscles under the control of *BG57-Gal4* driver (G-I, M-O).
A-C) Control muscles stained for DVAP (red) and SERCA, an ER/Sarcoplasmic Reticulum marker (Sanyal et al. 2005) (green) shows a good co-localisation.
D-F) Wt larval muscle stained with anti-Boca (green), specific for the ER (Culi and Mann 2003), reveals intense immunoreactivity around the nucleus and throughout the cytoplasm with again a good degree of colocalisation with DVAP (red).
G-I) *DVAPT48I* transgenic muscles stained with anti Boca (green) and DVAP antibody (red) shows brightly immunoreactive ER fragments which colocalize with DVAP-positive aggregates.
J-L) Control muscles stained with anti-Hsp70 (green) and anti-DVAP (red). The HSP70 immunoreactivity is under the detection limit of our antibody (J).
M-O) *DVAPT48I* transgenic muscles stained with anti HSP70 (green) and anti-DVAP (red). In these muscles there is an increased intensity of HSP70 staining, localised mainly to the nuclei, suggestive of an upregulation of HSP in the muscles of transgenic larva. Relocation of HSPs to the nucleus has been linked to cellular stress. Scale bars are 10 μm.

In wt *Drosophila* larva, DVAP immunoreactivity is distributed throughout the cytosol of the muscle with areas of increased intensity evident particularly around the nuclei of the muscle. There is a high degree of colocalisation with SERCA, a Calcium ATPase that is localised at the sarco-endoplamsic reticulum in *Drosophila* muscles and Boca, an ER marker. Similar to the expression of *DVAPT48I* in neuronal cells, in muscles expressing the mutant transgene, aggregates are present and the staining for Boca is disrupted, which could be indicative of fragmentation of the ER. Similar to the previous staining in the larval brain, there is extensive colocalisation of the Boca Staining with the DVAP inclusions (See figure 3.6). Transmission Electron Microscopy should be carried out to confirm the exact effect of DVAPT48I on the ultrastructure of the cell and show whether the ER is truly fragmented. For now, the staining is consistent with the findings in a number of SOD1 models in which targeted expression of the mutant protein has a direct toxic effect on the muscle. This conclusion is supported by the evidence of an upregulation of HSP70 seen in the transgenic lines. Here there appears to be a re-localisation of HSP70 into the nucleus which is a phenomenon linked to cellular stress giving further evidence that muscles cells are stressed following the expression of *DVAPT48I*.

Research in ALS is finding more and more evidence to suggest that this is not simply a disease of the motor neurons but a multi-systemic disorder and my findings here support this hypothesis as the muscle of ALS patients will be undergoing cellular stress and possible degeneration in addition to the degeneration occurring in the motor neurons. The primary pathological event in ALS is yet to be discovered but such findings as we have here shed some light on the events that might be leading up to cell death.

3.8 Effect of *DVAPT48I* over expression in the adult eye

DVAPT48I has the ability to induce aggregate formation and disrupt the ER but it is unknown whether this also brings about degeneration in a manner similar to *DVAPP58S*. If human *VAPBT46I* is a true causative allele for ALS it would be expected to recapitulate a number of the major hallmarks of the disease when modeled in the fly and considering ALS, degeneration within the nervous system is obviously key to the disease process. For this reason, *DVAPT48I* expression was localised to the adult eye. Use of the *Drosophila* eye to model disease is a common technique that has proved a valuable tool in the understanding of degenerative disease (See section 1.5 for more information). Expression of the *DVAPT48I* transgene was driven by the eye specific driver *eyeless-GAL4* and the eye surface examined by scanning electron microscopy (SEM) (Figure 3.7).

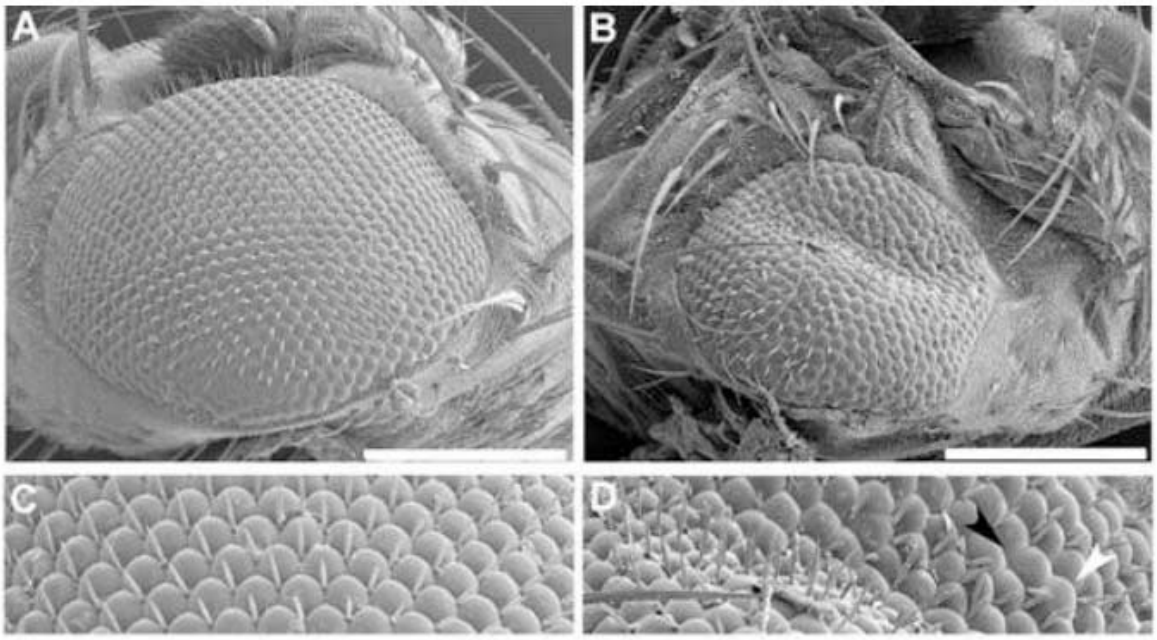


Figure 3.7 Targeted expression of DVAPT48I in the *Drosophila* eye causes a rough eye phenotype
Scanning electron micrographs of wt (A) and transgenic (B) adult fly eyes expressing the *UAS-DVAPT48I* transgene under the control of *eyeless-Gal4.* (C) and (D) are higher magnifications of (A) and (B) respectively. A decrease in size accompanied with missing bristles (black arrowhead) and fused ommatidia (white arrowhead) are seen when the *DVAPT48I* transgene is expressed in the eye.

The eye of the *Drosophila* adult is composed of an array of ommatidia with individual bristles arranged in an ordered and highly conserved pattern that can be easily screened under light microscope for changes. In flies expressing *DVAPT48I* the eye is much reduced in size and the neat ordered arrangement is lost to a rough eye appearance with missing bristles and fused ommatidia (see figure 3.7). Such a phenotype is consistent with neurodegeneration and a similar phenotype was also evident when the P58S transgene was expressed in the *Drosophila* eye (data not shown). This goes further to support the hypothesis that the *DVAPT48I* mutant allele is indeed causative for ALS and it is possible to speculate that the mutation affects DVAP in an identical way to the P58S mutation. To further support this idea, further analysis of the mutations on a biochemical level was investigated.

Chapter 4: Biochemical properties of mutant VAP vs wt

4.1 Homodimerisation of VAP proteins

To understand the role played by the mutant VAP proteins in ALS, an appreciation of the effect that the mutation has on the protein must be fully recognized. To begin such a characterization I focused upon the biochemical property of VAP proteins to interact with one another and form dimers, a process termed homodimerisation. The Yeast two hybrid system was used to investigate the deimerisation properties of VAP proteins which uses the yeast GAL4 transcriptional activator as described in Figure 4.1.

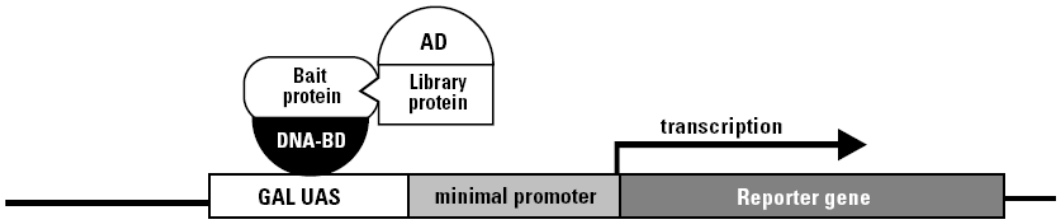


Figure 4.1 Yeast two hybrid system
The binding domain (BD) of the yeast GAL4 protein binds to the GAL4 UAS upstream of the reporter genes and the activation domain (AD) activates transcription of reporter genes. These two domains can be separated and fused to other proteins while maintaining their functionality. If brought into close enough proximity through the interaction of the proteins to which they have been fused they will activate the transcription of reporter genes thereby signaling an interaction within the yeast colony. Diagram taken from the MATCHMAKER GAL4 Two Hybrid System 3 and Libraries User Manual. (Clontech)

To investigate the ability of DVAP and human VAP to homodimerise, a number of small scale yeast two-hybrid experiments were carried out. The open reading frame for DVAP and hVAP were inserted into the yeast tranfection vectors pGBKT7 and pGADT7 which contain the sequence for the GAL4 binding domain and activation domain respectively. The resulting fusion proteins translated within the cell consist of VAP fused to an active domain of GAL4 and if a cell is cotransfected with genes inserted into both vectors then if the two resulting proteins interact, the two GAL4 domains are brought together to initiate transcription of reporter genes. The yeast cells were simultaneously transformed using the polyethylene glycol/lithium acetate

(PEG/LiAc)- mediated transformation protocol as outlined in the MATCHMAKER Two-Hybrid System 3 handbook and described briefly in Chapter 2.12. Following the transformation, cultures were plated on SD plates that are a combination of a Minimal SD Base and a Drop Out Supplement. This is a synthetic, defined minimal medium lacking one or more specific nutrients. Cells containing the pGADT7 vector alone can grow upon media lacking Leucine, Cells with pGBKT-7 can grow on media lacking Tryptophan and a cell that has been transformed with both vectors can grow on SD-Leu, -Trp plates. If the proteins expressed within a cell interact, the transcription of reporter genes means that the cells can grow on plates lacking Adenine and Histidine as well as Leucine and Tryptophan. Colonies will also be blue in colour due to the activity of α-galactosidase under the control of the *MEL1* reporter gene. In parallel with the experimental test transformations, yeast cells were also inoculated with control plasmids supplied by Clontech. PGADT7-T and pGBKT-53 contain the known interacting proteins SV40 large T-antigen and murine p53 respectively and act as the positive control. PGADT7-T and pGBKT7-Lamin C are known to not interact and act as a negative control.

The experimental set up was as follows:

pGADT7 wt DVAP and pGBKT7 wt DVAP
pGADT7 mut P58S DVAP and pGBKT7 mut P58S DVAP
pGADT7 mut DVAP and pGBKT wt DVAP
pGADT7 wt VAPB and pGBKT wt VAPB
pGADT7 mut P56S hVAPB and pGBKT7 mut P56S hVAPB
pGADT7 mut P56S hVAPB and pGBKT7 wt hVAP B
pGADT7 mut T48I DVAP and pGBKT7 wt DVAP
pGADT7 mut T48I DVAP and pGBKT7 mut T48I DVAP

Finally, the human protein VAPB was extensively studied in *Drosophila* by expressing the wt and mutant VAPB in a wt background and when considering the phenotypic results it is interesting to see how the interactions between the VAP proteins is affected

by the inter-species differences in sequence. The human protein had the ability to rescue the phenotype of a DVAP null mutant so the two proteins are functionally interchangeable as well as homologous on the level of genetic sequence but this does not necessarily mean they will act in the same manner when in the presence of the endogenous wt protein. For this reason the following binary interactions were also carried out.

pGAD-hVAP and pGBKT7 DVAP

pGAD-Mut hVAP and pGBKT7 DVAP

4.2 Results from the binary Yeast two-hybrid interactions

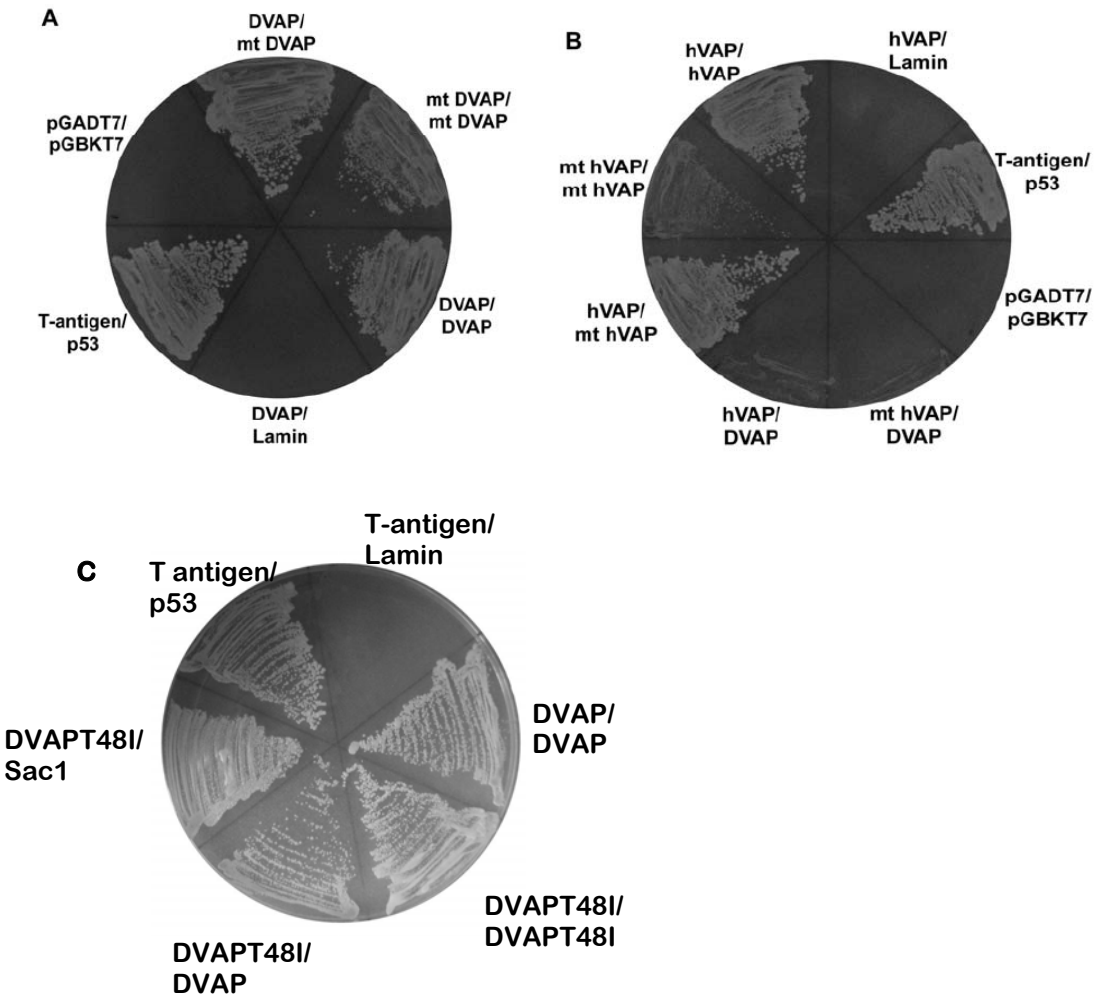


Figure 4.2 Homodimerisation of VAP proteins is not affected by the mutations
Plates A, B and C show co transformed yeast taken from low stringency plates lacking Leucine and Tryptophan and re-plated onto high stringency plates also lacking Adenine and Histidine in addition to Leucine and Tryptophan (SD-AHLT). Yeast co-transformed with pGADT7 and pGBKT7 empty cloning vectors act as a negative control as do yeast co-transformed with pGAD-VAP and pGBKT-Lamin. PGBKT-53/pGADT7-T acts as a positive control and shows good growth on the high stringency plates. A and B) Mutant proteins carry the original P56S/P58S mutation. C). The examination of the ability of DVAPT48I to interact with wt DVAP and homodimerise. This plate also shows the interaction between Sac1 and DVAP T48I discussed in section 5.

Table 4.1	DVAP-33A	P58S mut DVAP-33A
DVAP-33A	+	+
P58S mut DVAP-33A		+

Table 4.2	Hum VAP B	P56S mut hum VAPB
Hum VAPB	+	+
P56S mut Hum VAPB		+

Table 4.3	DVAP-33A	T48I DVAP-33A
DVAP-33A	+	+

Table 4.4	Hum VAP B	P56S mut hum VAPB
DVAP-33A	-	-

Table 4.1,2,3 and 4. Summary of VAP homodimerisation results
Summary of the results from a number of small scale yeast 2-hybrid experiments looking at the binary interactions of the *Drosophila* and human VAP proteins. + shows an interaction, - no interaction was observed.

4.3 Homodimerisation is not affected by P58S/T48I mutations

Results from these experiments indicate that DVAP has the ability to homodimerize (Figure 4.1). Yeast containing pGADT7-DVAP and pGBKT-DVAP grew well on the high stringency plates that lacked Adenine, Histidine, Leucine and Tryptophan (SD-AHLT) and the colonies were blue in colour. Dimerisation is likely to be crucial for the activity of DVAP and it is therefore important that both of the mutant DVAPs can also

homodimerise as it demonstrates that the mutation does not interfere negatively on this biochemical property. The aggregates seen in the *Drosophila* model of ALS8 are believed to be composed of both wild type and mutant protein and the interaction between these two proteins will be necessary for the aggregate formation. Consistent with this, yeast co-transformed with either DVAP mutant and wild type DVAP show good growth on the SD-AHLT plates.

The ability of human VAPB to rescue the phenotype of a null *Drosophila* mutant indicates that the two proteins are functionally interchangeable. If dimerisation is key to the function of DVAP it is very likely to be conserved in the human homologue and the results of this yeast two-hybrid show this to indeed be the case for both wild type and mutant hVAPP56S.

In contrast to these positive results, when the ability of hVAPB to interact with DVAP was examined, no interaction was evident and nothing grew on the high stringency plates despite the presence of colonies on the low stringency plates selecting for co-transformation. The lack of interaction, especially of the mutant hVAPB with DVAP, is particularly intriguing considering the decision to model the disease in *Drosophila* by expressing a mutated form of the endogenous DVAP protein rather than the human VAPB. Expression of VAPBP56S in a *Drosophila* wild type background gives rise to a similar phenotype as that seen by the overexpression of wt DVAP and does not show the extensive hallmarks of disease that our DVAPP58S *Drosophila* model presents with. One such hallmark is the presence of aggregates and we believe that the formation of these large insoluble inclusions, incorporating both wt and mutant VAP, sequester the wt endogenous protein away from its normal localisation. This gives rise to a dominant negative situation and the mutant protein causes a loss of function phenotype despite it having the ability to rescue the phenotype of a null mutant in which no endogenous DVAP is present. The interaction between the mutant and wt protein is key to this aggregation and the inability of hVAP and DVAP to interact would account for the lack of this hallmark and others present in the model. This warns of the potential problems that might arise when modeling any disease in *Drosophila* through the expression of a human protein. This is a common practice that has produced many

invaluable models for studying human disease but it is certainly worthy of note as in this case the human protein appears to be functionally interchangeable with DVAP in a null fly but is incapable of modeling the disease when expressed with wt endogenous protein of *Drosophila*. Co-immunoprecipitation is currently being carried out in the lab to confirm the yeast two hybrid results and preliminary results are in support of the data from the Yeast two hybrid system.

Chapter 5: Effect of the VAP mutations on complex formation

5.1 Interaction of VAP with Sac1

Another potential effect of the pathogenic mutation is to disrupt the interaction of VAP with other proteins in the cell and therefore affect the complex formation of VAP. An interactor of DVAP as noted in a large scale genome wide screen by Giot *et al* is Sac1 (Giot *et al.* 2003). Sac1 is a lipid phosphastase that is necessary for the regulation of a pool of $PI_{(4,5)}Ps$ and is thought to play a role in a number of functions including cell morphology and the organization of the cytoskeleton (Foti *et al.* 2001; Wei *et al.* 2003a; Liu *et al.* 2008a). Considering the observed role of DVAP in homeostatic control of the NMJ with regard to the size and number of synaptic boutons (Pennetta *et al.* 2002), an interaction with Sac1, that too elicits changes in cell morphology, makes this an interesting interaction. To verify this interaction and look at the ability of Sac1 to interact with the wild type and mutant forms of DVAP a small scale yeast two-hybrid was carried out. It is important to understand the effect of the mutation on the interactions formed by VAP proteins as a loss of an interaction will affect the normal function of such proteins and will extend our knowledge of the pathways affected. Maintenance of an interaction could mean that the protein is incorporated into the aggregates and similar to the reduction in endogenous VAP from its original location, it might also sequester the interacting protein. The ability of hVAPB to interact with Sac1 was also investigated as human VAP is functionally interchangeable with DVAP, so if this is an important interaction it might be maintained despite Sac1 being a *Drosophila* protein. The cDNA for Sac1 was obtained from the DGRC and the open reading frame subcloned in frame into the yeast transformation vector pGBKT7. Yeast was co-transfected with pGADT7-DVAP and pGBKT7-Sac and the transformed yeast plated onto medium lacking Adenine, Histidine, Leucine and Tryptophan. Growth on these plates will indicate an interaction.

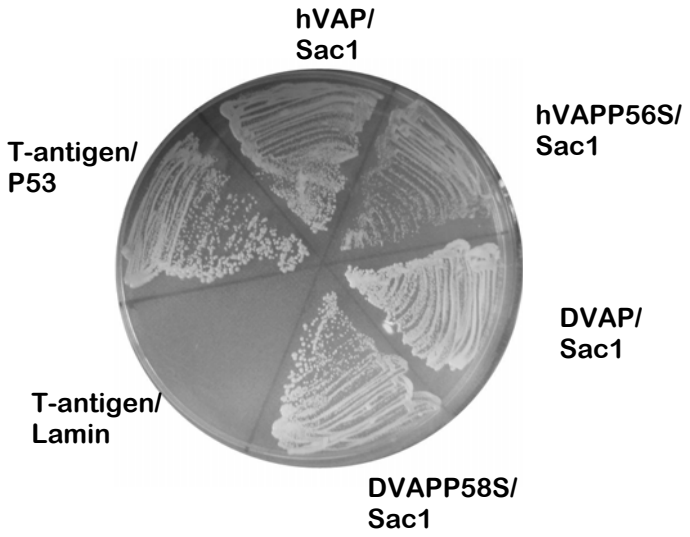


Figure 5.1 Interaction of VAP and Sac1 is not affected by the mutation
Plate showing co transformed yeast taken from low stringency plates lacking Leucine and Tryptophan and re-plated onto a high stringency plate also lacking Adenine and Histidine in addition to Leucine and Tryptophan (SD-AHLT). Yeast co-transformed with pGADT7 and pGBKT7-Lamin act as a negative control. pGBKT-53/pGADT7-T acts as a positive control and shows good growth on the high stringency plates.

	DVAP	P58S DVAP	T48I DVAP	VAP B	P56S VAPB
SAC1	+	+	+	+	+

Table 5.1 Summary of the VAP-Sac1 yeast two hybrid results
Summary of the results from a small scale yeast 2-hybrid looking at the binary interactions of the DVAP and hVAP with the Drosophila SAC1 protein.

Yeast co-transformed with either wt or mutant DVAP and Sac1 grew well on the high stringency AHLT plates which indicates that despite the mutation or species of origin, VAP proteins are able to interact with the lipid phosphatase Sac1 (figure 5.1 and figure 4.1 for the plates showing the DVAPT48I/Sac1 interaction). A confirmation of this interaction for wt DVAP and DVAPP58S was carried out by Co-IP. The cDNA for Sac1 was obtained from the DGRC and subcloned into pCMV-myc in order to express Sac1 in COS-7 cells under the control of the cytomegalovirus promoter. After two days

the cells were lysed and the lysate incubated with α-Myc beads overnight. A wash buffer containing Digitonin was used to remove non-specific binding to the beads and the presence of interacting proteins was detected by Western blot (see figure 5.2 below). It should be noted that these are preliminary data and experiments are ongoing in the lab to improve the technical quality of these results. However these preliminary data show a good interaction of DVAP with Sac1 which is maintained despite the disease mutation in DVAP.

5.2 Confirmation of Yeast two hybrid results by Co-IP.

Figure 5.2 Confirmation of DVAP-Sac1 interaction by Co-IP
Western blots from a co-immunoprecipitation carried out with COS7 cells co-transfected with either wtDVAP (A and B) or mutant DVAPP58S (C and D) and Myc tagged Sac1 Anti Myc agarose beads were used to bind the Myc tagged Sac1 and any other interacting proteins. A and B) Anti DVAP staining for the presence of wt DVAP in the input (I) and a strong clear band in the test lane (Pellet $P_{dVAP\ Sac1}$) confirms that wt DVAP can interact with Sac1. In these western blots the Input from the two control samples was not run but the supernatant shows any remaining protein that did not bind to the beads. B) Anti-Myc staining for the presence of Sac1-Myc in the input ($I_{dVAP\ Sac\ 1}$) and attached to the beads ($P_{\ dVAP\ Sac1}$ and $P_{\ Sac1}$). There is no protein staining visible in the supernatant lane as all Sac1-Myc is attached to the beads. C and D) Anti DVAP again shows a strong band in the test lane Pellet $P_{P58S\ Sac1}$ indicating that in agreement with the Yeast two hybrid results, mutant DVAP retains its interaction with Sac1. D) Anti-Myc staining for the presence of Sac1-Myc in input ($I_{\ P58S\ Sac1}$) is very faint but there is a very strong band showing the attachment to the beads ($P_{\ P58S\ Sac1}$ and $P_{\ Sac1}$).

5.3 Interaction with SAC1 is not affected by mutation

Both human and *Drosophila* VAP can interact with the *Drosophila* Sac1 protein. Yeast transfected with pGBKT-SAC1 and either wt or mutant P56S/P58S VAPB or DVAP grew well on the high stringency plates that lacked Adenine, Histidine, Leucine and Tryptophan and the colonies were blue in colour. This was also true for yeast transfected with *DVAPT48I* and Sac1 (as shown in earlier figure 4 plate). The Co-IPs further confirm these results. Sac1 proteins have 7 highly conserved domains that are evident in all species from yeast to man and if the interaction utilizes one or more of these conserved domains the ability of this *Drosophila* protein to interact with the human VAPB is understandable. As the mutant forms of DVAP and VAPB can rescue the phenotype associated with a knock out of the endogenous protein, the important complexes may still be maintained and these results are consistent with this. The Sac domain is present in a number of proteins including SAC3, described previously as the causative gene for ALS11. The ability of VAP to interact with other SAC domain proteins is an important avenue for further investigation that could answer many questions about the role of VAPs and shed light on how the P56S/T46I mutation causes neurodegeneration. It should be considered that the mutation may cause an increase in the strength of interaction. The data reported here are not quantitative and both for the Sac1 interaction and the homodimerisation of VAP, there may be an increased propensity to interact with the mutant protein. The result from this might be the formation of aggregates as seen following the expression of DVAPP56S/DVAPT48I in the fly and COS7 cells

The next intriguing question is whether Sac1 is directly involved in the degenerative phenotype associated with mutant VAP. This could be due to Sac1 being sequestered away within the aggregates and away from its wt localisation or with the formation of aggregates, DVAP may be unable to localize with SAC1 and despite mutant VAP having the ability to interact, spatial parameters prevents this. Before continuing with such investigations, an examination of the domains necessary for the

VAP-SAC interaction was carried out to see whether the residues necessary for interaction are affected by the mutation.

5.4 Narrowing down the interacting domains for VAP1 and Sac1

In order to deduce the regions necessary for the interactions reported earlier, constructs were designed and cloned which contained shorter sequences of Sac1 and DVAP. By removing domains from each protein and looking at the ability of these truncated proteins to interact it should be possible to deduce which regions are important for the interaction.

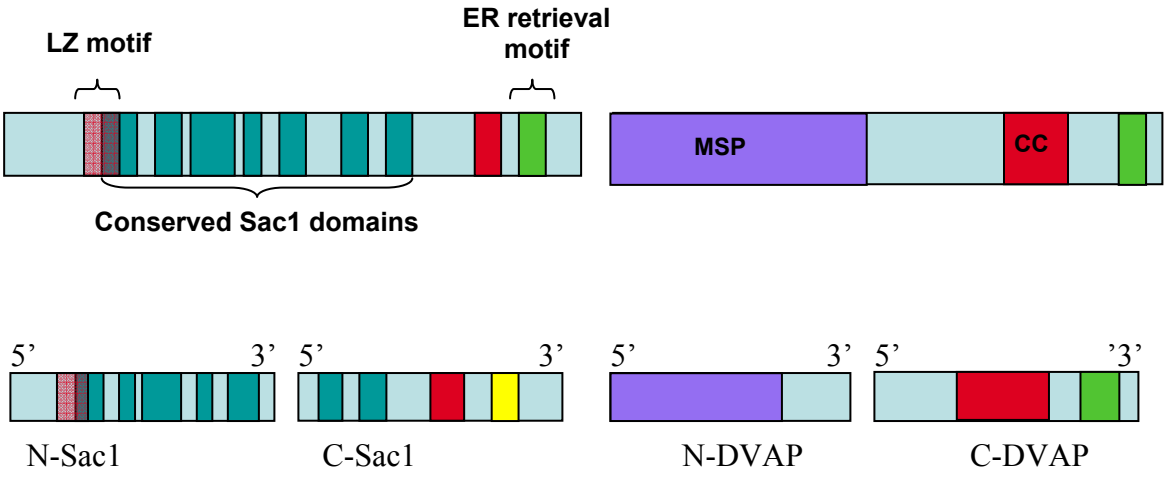


Figure 5.3 Truncated proteins used in yeast two hybrid
Diagrammatic representation of the truncated proteins used in the binary yeast two hybrid aimed at narrowing down the regions necessary for interaction. Pink represents the LZ domain of Sac1. Blue the Sac domains. Yellow: ER targeting motif. Purple: the MSP domain of DVAP. Red: coiled coil domain and green: TM domain.

Nterm DVAP. First 417 base pairs of the DVAP open reading frame ligated into the pGADT7 vector. Resulting protein contains 139 amino acids and includes the MSP domain.

Cterm DVAP. Final 411 base pairs of the DVAP open reading frame ligated into the pGADT7 vector. Resulting protein contains 137 amino acids and includes the predicted coiled-coiled domain and the transmembrane domain (Chai *et al.* 2008).

Nterm Sac1. First 1050 base pairs of Sac1 open reading frame ligated into the pGBKT7 vector. Resulting protein contains 500 amino acids and includes the predicted LZ motif and the first 5 of the 7 conserved Sac1 domains (Hughes *et al.* 2000).

Cterm Sac1. Final 774 base pairs of Sac1 open reading frame ligated into the pGBKT7 vector. Resulting protein contains 358 amino acids and includes the final 2 conserved Sac1 domains, the transmembrane domain and the C terminal ER retrieval motif.

The cDNA for these subfragments were amplified by PCR and cloned into the yeast two hybrid transformation vectors PGBKT and pGADT7 and a small scale Yeast two hybrid carried out. There is some discrepancy in literature as to which regions are necessary for homodimerisation. Investigating which of these truncated DVAP proteins will be able to interact with full length DVAP or Sac1 will be interesting when considering the function played by the mutations in the MSP domain

5.3.1 Results for the truncated protein yeast two hybrid interactions

Table 5.2	DVAP	SAC1
N-DVAP	-	-
C-DVAP	+	+

Table 5.3	DVAP
N-SAC1	-
C-SAC1	-

Table 5.2 and 5.3. Summary of the yeast two hybrid results looking at the interaction with truncated proteins
Tables summarising the ability for the shortened DVAP and SAC1 constructs to interact with the full length proteins.

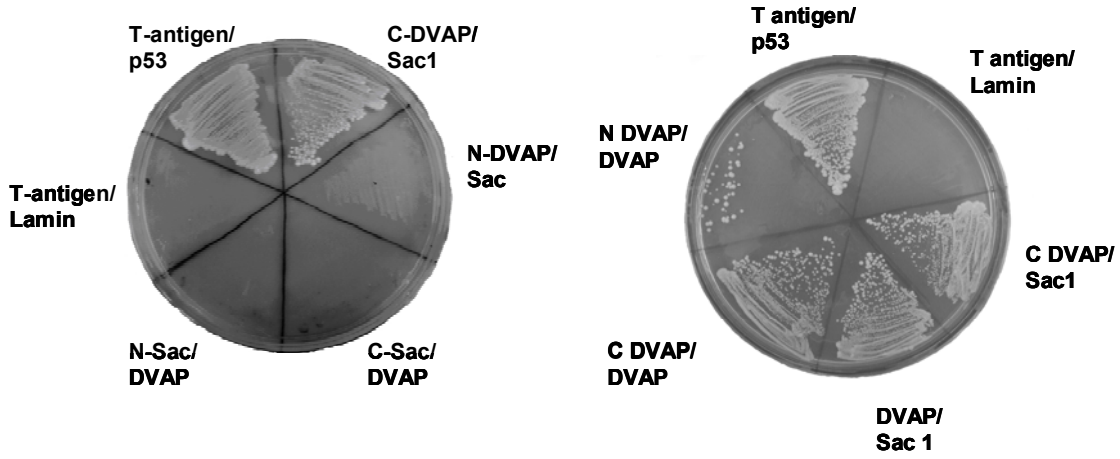


Figure 5.4. Plates from yeast two hybrid looking at the interaction of truncated proteins
Plates show co transformed yeast taken from low stringency plates lacking Leucine and Tryptophan and re-plated onto high stringency SD-AHLT plates. Yeast co-transformed with pGADT7 and pGBKT-Lamin constructs act as a negative control as do yeast co-transformed with pGAD-VAP and pGBKT-Lamin. PGBKT-53/pGADT7-T acts as a positive control and shows good growth on the high stringency plates.

5.3.2 MSP domain is not necessary for homodimerisation or interaction with Sac1

Results from these experiments indicate that only the final 136 amino acids of DVAP-33 are required both for the interaction with Sac1 and itself. Yeast transfected with C-DVAP and either full length DVAP or Sac1 grew well on the high stringency plates that lacked Adenine, Histidine, Leucine and Tryptophan and the colonies were blue in colour. As controls in this experiment PGADT7-T and pGBKT-53 also showed an interaction as did the full length DVAP and Sac1. Similar to the negative control of Lamin C and T antigen, neither of the shortened Sac1 constructs interacted with DVAP nor did the N-DVAP construct interact with Sac1.

The division of the Sac1 domains in both the Sac1 constructs designed above may be the cause of a lack of interaction. It is possible that all seven domains are important for protein interactions. As such, another Sac1 construct was designed containing all seven of the conserved Sac1 domains and missing only the Transmembrane domain and the ER motif. At a length of 1455 base pairs, the transcript encodes a protein of 485 amino acids.

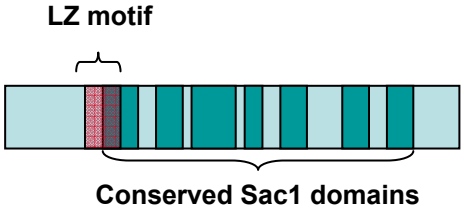


Figure 5.5. Diagram of Sac1 truncated protein containing all 7 conserved Sac1 domains

The above fragment was cloned into pGBKT7 and transfected into yeast along with pGADT7-DVAP but again no interaction was observed suggesting that the whole of the protein is necessary for interaction or at least for stability. It is possible that the truncated protein is being degraded by the yeast cell and this should be tested either by western blot or repeating the experiment in another system such as cell culture.

Chapter 6: Investigation of Sac1 in the Nervous System

6.1 VAP interacts with Sac1

Data has been presented that verifies the interaction of DVAP and hVAPB with Sac1. This has the potential to be a very important interaction considering the identified role of Sac1p in yeast in cytoskeletal organization and cellular morphology. Wei *et al* have shown that Sac1 is vital for dorsal closure which involves the migration of the lateral epidermal flanks over the amnioserosa driven by the actomyosin contractile apparatus (Wei *et al.* 2003a). There is a dramatic morphological change in the shape of the cells during this process and a link with Sac1 means that this protein could be a key player in regulating the changes observed at the synaptic boutons of DVAP mutants. The down stream effectors of Sac1 may also be responsible for the changes in synaptic bouton morphology in reponse to dosage of VAP at the NMJ. In addition to this, mutations in *SAC1* have been found to enhance the degenerative eye phenotype seen in the fly model of spinocerebellar ataxia 8 (SCA8) (Mutsuddi *et al.* 2004) (see introduction for a more detailed description of this model). Further analysis of the role played by Sac1 in the fly is required in order to elucidate the involvement in the mutant phenotype associated with DVAP.

There are already a number of mutants available for Sac1 in *Drosophila* but unfortunately due to the requirement in dorsal closure, null mutants are embryonic lethal which makes a functional analysis of the neuromuscular junction impossible. The positions of the mutants available from Flybase are shown in Figure 7.1 below.

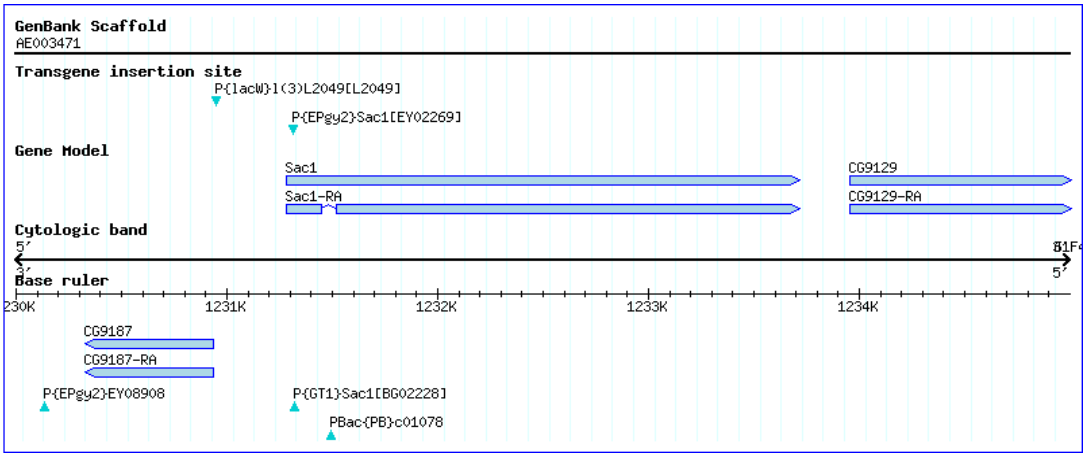


Figure 6.1 Genomic region of Sac1
Sac1 is present on chromosome 3. This diagram indicates the position of available P-element inserts available within this region. (Flybase).

The three mutations available from the *Drosophila* stock centre are 1) P(GT1)Sac1[BG02228], 2) PBac(PB)c01078 and 3) (EPgy2)Sac1[EY02269]. The PBac(PB)c01078 (2) is inserted in the first intron of the 5' untranslated region, 60 base pairs upstream of the AUG and mutant flies survive to adulthood. P(GT1)Sac1[BG02228] (1) is in the first exon, 234 base pairs upstream of the AUG and is the mutant line used by Wei *et al* and is embryonic lethal. Finally P(EPgy2)Sac1[EY02269] (3)n is in the first exon of the 5' UTR, 242 bp upstream of the AUG and the few escapers in this stock die at the first instar larval stage due to problems of dorsal closure (Wei *et al.* 2003a). Attempts were made to rear these larvae to a later stage as it has previously been shown that larvae carrying apparently embryonic lethal mutations in synaptotagmin can be reared to adulthood by optimizing the conditions of culture (Loewen *et al.* 2001). By removing the homozygous larvae from competitive heterozygous siblings and providing a thinner yeast paste that is easier for them to consume, the problems in mobility are overcome. Such conditions were of little help to the EY02269 flies as they have severe developmental problems underlying death. Wei *et al* had a hypomorphic allele that promoted defective eye development but this has unfortunately been lost (Wei *et al.* 2003a) .

Following the identification of Sac1 as an interactor of DVAP the function of this protein in the fly deserves to be further investigated. Unfortunately these mutant stocks are not ideal for investigation as they are embryonic lethal or as in the case of PBac (PB)c01078 have little evident phenotype other than a potential sterility problem. This PBac stock has further problems in that a few flies always have white eye which indicates a loss of the transgene. Considering all this the first step is to characterize the phenotype associated with an over-expression of the protein This was achieved through the creation of *UAS-SAC1* transgenic lines that express the *SAC1* gene under the control of the pan neural *elav-Gal4* driver. The cDNA for *SAC1* was obtained from the BDGP gold cDNA collection from *Drosophila* resource centre and this was subcloned into the pUAST. The construct was injected into *Drosophila* embryos and a number of transgenic lines were established. Third instar larvae from the transgenic lines (*elav;UAS-SAC1*) were dissected and neuromuscular junctions stained with the HRP antibody.

As the Sac1 null flies created by Wei *et al.* were shown to be lethal due to the necessity of Sac1 in dorsal closure our ability to look at the nervous system by dissection was greatly impeded. To overcome this problem we undertook the task of creating a loss-of-function mutation as a dominant negative which will act as a functional null in the areas of expression i.e. the larval nervous system, meanwhile leaving wt Sac1 levels unchanged in other tissue (thereby bypassing the first requirement for the gene). The production of a dominant negative was proposed by the removal of a suspected vital region of the Sac1 sequence necessary for its function. The resulting mutant protein would compete with the wild type and inhibit its action thereby producing a hypomorph. An RNAi stock was also procured from the Vienna *Drosophila* Resource Centre (VDRC). Utilising the spatial and temporal control provided by the UAS/GAL4 system makes it possible to drive the Sac1 dominant negative allele and RNAi solely in the larval nervous system. This will overcome the lethality due to the necessity of Sac1 in embryonic gastrulation as only where the mutant protein is driven will the wild type Sac1 be affected. This may give a phenotype suggestive of Sac1's role at the synapse.

6.2 Creation of a SAC1 Dominant Negative

A number of proteins contain a Sac domain comprising of seven highly conserved motifs (see Figure 6.2 below). The sequence RTNCIDCLDRTN is particularly highly conserved and contains the $CX_5R(T/S)$ motif present in a variety of metal-independent protein and inositide phosphatases that have been well characterized. This conserved motif is believed to cradle the phosphate moiety necessary for the catalytic action of these proteins and perhaps the Sac1 proteins as well (Guo *et al.* 1999; Hughes *et al.* 2000). The yeast proteins Sac1p, Inp52p, Inp53p, and synaptojanin are all active phosphatases whereas Inp51p, which differs only by three residues in this putative catalytic motif, has no phosphatase activity adding weight to this hypothesis (Hughes *et al.* 2000). Finally there are a number of mutant yeast strains for Sac1p that lack the phosphatase activity, one being *sac1-8* in which the first Aspartate residue is mutated in the $CX_5R(T/S)$ motif (Nemoto *et al.* 2000).

The coding region of the Sac1 gene was obtained from Flybase and was aligned with the yeast Sac1p (NCBI) and the catalytic motif was found to be conserved in the *Drosophila* Sac1. This is a good candidate region to mutate to produce a dominant negative mutant and two options were considered. First a deletion of the entire DNA sequence encoding the motif RTNCIDCLDRTN. Second was to change only the first Aspartate residue in this sequence found to be important both in yeast and also in rat (Hughes *et al.* 2000; Nemoto *et al.* 2000). The change of a negatively charged Aspartate residue to a positive Asparagine should be enough to disrupt the catalytic activity whilst maintaining the normal folding such that it successfully competes with the wild type protein. Site directed mutagenesis was carried out to remove/ change the necessary sequence and the resulting cDNA subcloned into the transformation vector pUAST ready for injection. A number of *Drosophila* transgenic lines were established.

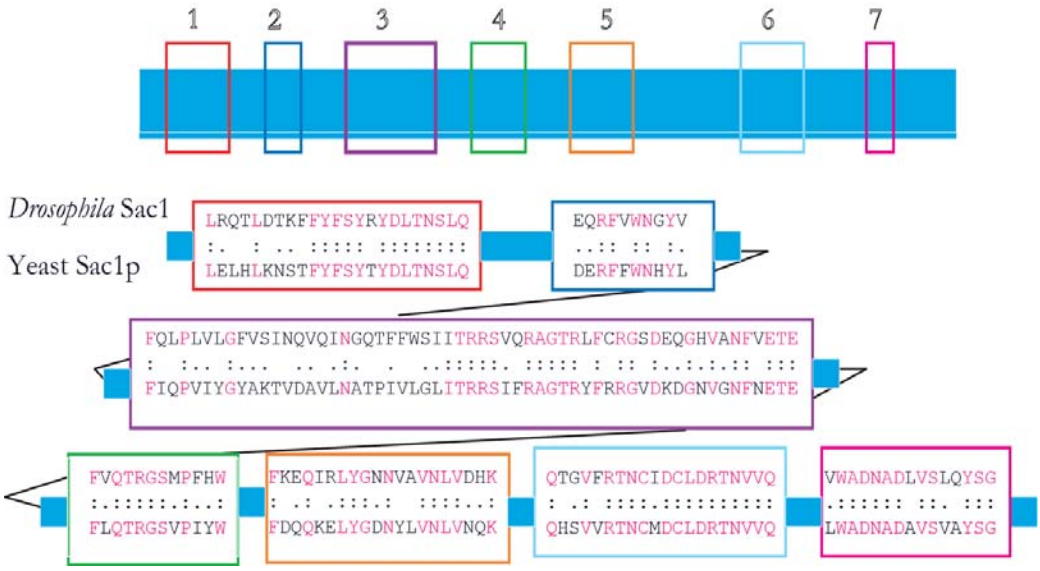


Figure 6.2 Alignment of the *Drosophila* Sac1 gene with the yeast Sac1p.
There are 7 conserved SAC1 motifs and the 6th is the predicted catalytic domain. Adapted from Hughs *et al* 2000.

6.3 Effect of Sac1 dosage at the neuromuscular junction

The role of Sac1 at the NMJ has never been investigated and considering the aforementioned role of Sac1 in cellular morphology, a good starting point in the investigation of Sac1 and VAP is to look at the effect that Sac1 overexpression and under expression has at the NMJ. Larvae expressing Sac1 RNAi driven pan-neurally by the *elav-GAL4* driver (Yao and White 1994) (*elav; SAC1RNAi)* were dissected and the NMJ stained for the neuronal cell surface marker HRP. Transgenic larvae overexpressing the wt *SAC1* under the control of *elav-GAL4 (elav; UAS SAC1)* were also dissected and the pattern of staining in these two lines, as compared to controls, was analysed at muscle 12 and 13 of the larval NMJ. (Figure 6.3).

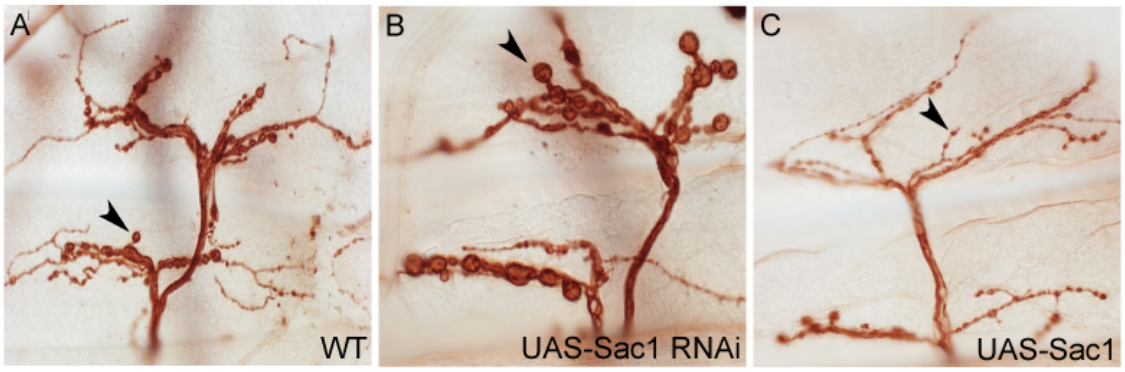


Figure 6.3 Effect of Sac1 dosage on bouton formation at the NMJ
Effect of dosage of Sac1 was examined at muscle 12 and 13 of flies injected with a *UAS-SAC1* construct and the transgene expression driven by the pan neural *elav-GAL4* driver. The UAS-Sac1 RNAi flies were also crossed with an *elav-GAL4* driver to express the transgene and thereby reduce the expression of Sac1 pan-neurally. Pictures obtained from Andrea Chai.

An over-expression of Sac1 at the NMJ of third instar larvae is associated with a decrease in bouton size and an increase in bouton number. Similarly, a decrease in Sac1 level by the expression of a *UAS-Sac1 RNAi* leads to an increased bouton size with an apparent decrease in number. Preliminary observations of flies expressing the dominant negative Sac1 suggests that results are similar in these flies too. A statistical analysis is yet be carried out but the observed changes are dramatic and parallel those seen for DVAP in which significant changes in bouton size and number are observed following the over expression or knock- out of DVAP in the nervous system of larvae (see Figure 6.4 below (Chai *et al.* 2008). In conjuction with the yeast two-hybrid data the results point towards a common pathway for Sac1 and DVAP in their ability to affect the morphology of the synapse.

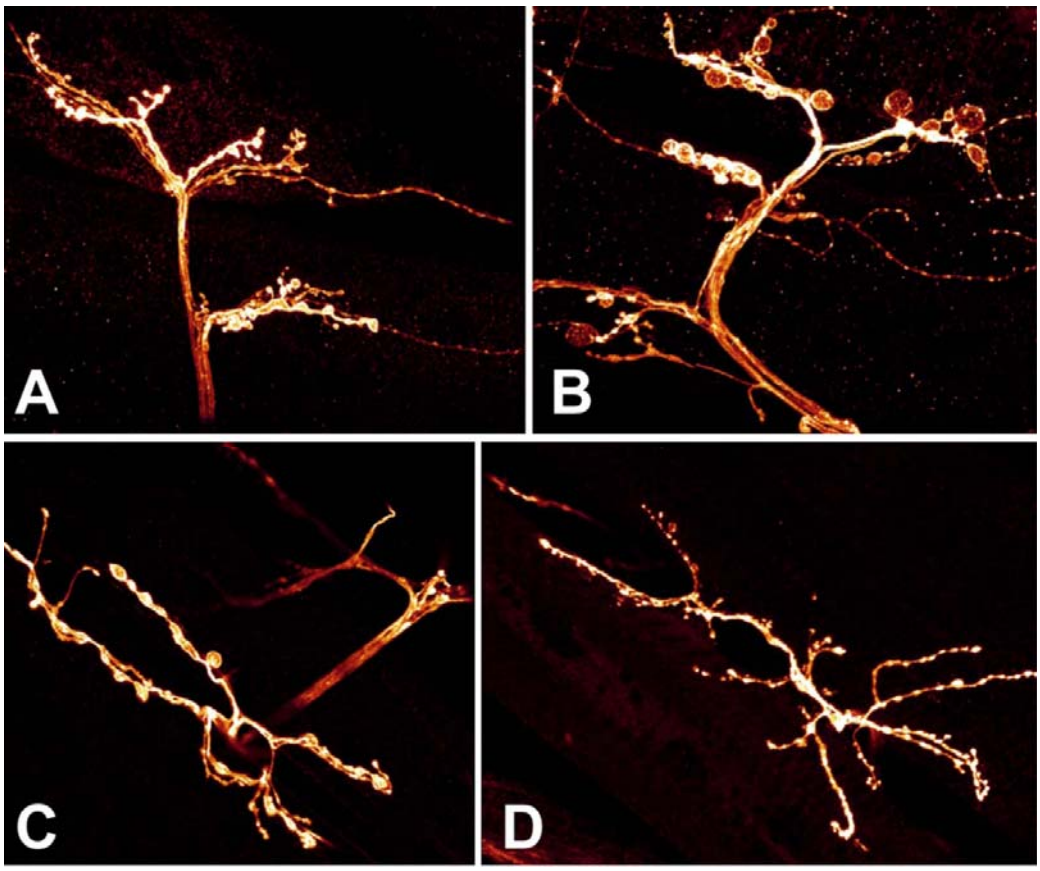


Figure 6.4 Dosage dependent effect of DVAP at the NMJ
Dosage dependent effect of DVAP on the synaptic boutons of muscles 12 and 13 at the larval NMJ stained with anti HRP. (A-C) Wt controls. B) $DVAP_{\Delta 166}$ null mutant has fewer boutons that are of a larger size as compared to wt. D) UAS-DVAP over expressing larval NMJs have many more boutons that are reduced in size. Images from Chai *et al* 2008.

6.4 Effect of Sac1 on the *Drosophila* eye

A Sac1 mutation has previously been shown to enhance the degenerative phenotype of *SCA8* in a *Drosophila* model of the disease which utilizes the eye as a model. To further investigate the role played by *SAC1* in degeneration, the effect of a reduction in *SAC1* expression on the formation of the eye was investigated. Flies from the pan neural expression of RNAi SAC1 transgene showed necrotic patches in the eye (Figure 7.5). Interestingly, because the *elav-gal4* driver is inserted on the X chromosome, it is possible to see that Sac1 has a dosage dependent effect on neurodegeneration because

males have a stronger phenotype. This is linked to an upregulation due to the dosage compensation of the X chromosome in males which leads to an increased expression of GAL4 in the males flies. In the females, although milder, there is a degenerative phenotype characterized by depigmentation and roughness. Necrotic patches are seen in the eye of l(3)2107 mutant species which also disrupt the *SAC1* gene (Wei *et al.* 2003b). Unfortunately this fly line is no longer available. Necrotic patches are indicative of cell death and this provides a tentative link between Sac1 and the neuronal death seen in our fly model of ALS which should be further investigated.

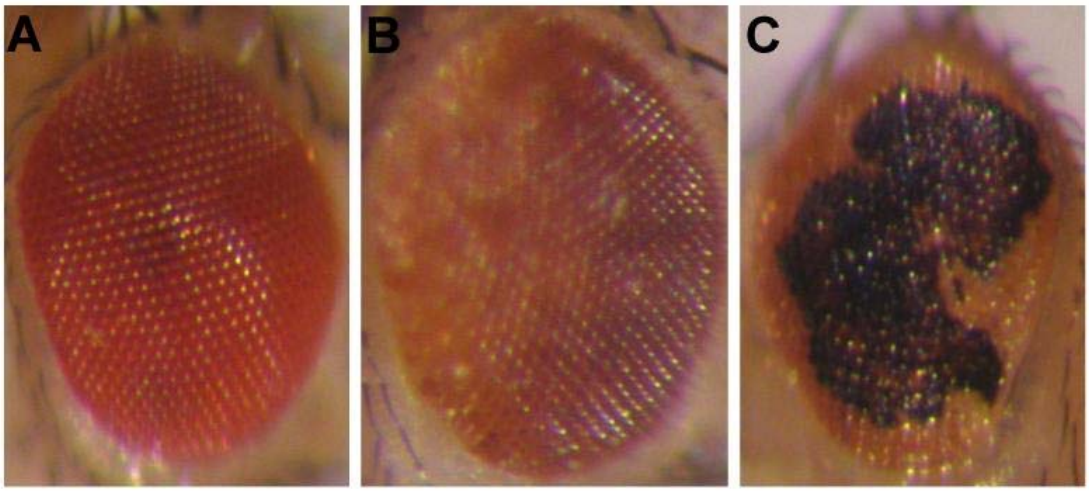


Figure 6.5 Necrotic patches in the eye of *UAS-SAC1 RNAi* flies.
Flies expressing *UAS-SAC1* RNAi under the control of a pan neural driver show a dosage dependent development of necrotic patches in the eye. A) wild type eye. B) Eye of a female expressing RNAi SAC1. C) Male eye expressing RNAi SAC1. The driver regulating the expression of the transgene is on the X chromosome which means that the expression is influenced by the dosage compensation seen in males.

The eye phenotype was investigated further by the eye specific reduction of Sac1 which was established by expressing the RNAi Sac lines under the control of the driver *elav-Gal4*. The morphology of the surface of the eye was observed using scanning electron microscopy (SEM) See Figure 6.6. The eye specific reduction in SAC1 causes a reduction in eye size, a severe rough eye phenotype with a reduction in eye size, fused ommatidia and missing bristles. This is all indicative of degeneration of the photo receptor cells and is similar to the eye phenotype seen when *DVAPP58S* is expressed in the eye.

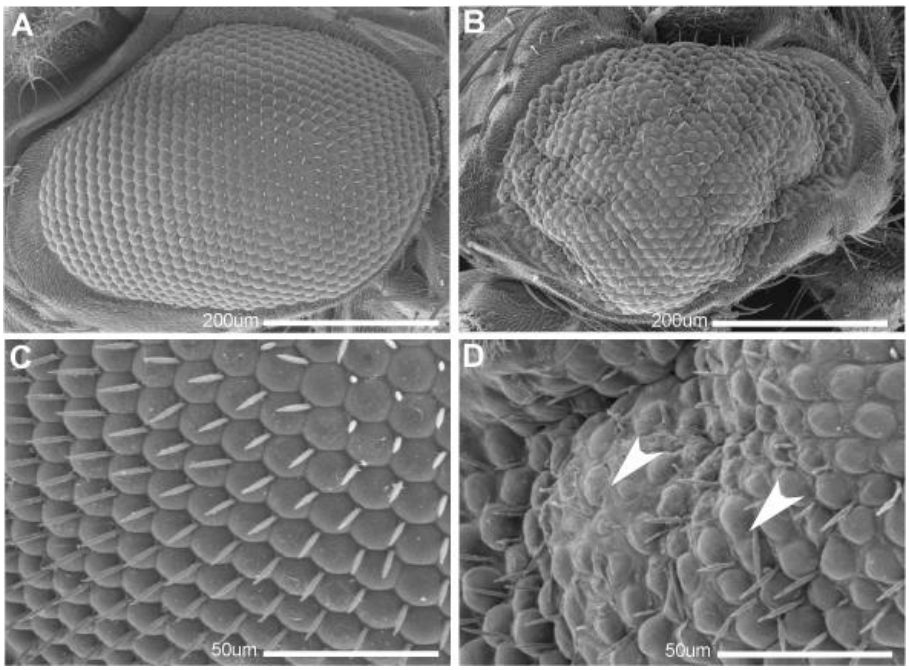


Figure 6.6 Eye specific expression of SAC1 RNAi (panel B and D) show a severe rough eye phenotype
B and D) Eye specific expression of SAC1 RNAi induces a severe rough eye phenotype accompanied by a decrease in the size and a disruption of the ommatidia as compared to the wild type eye (panel A and C). Arrowheads pinpoint areas of fused ommatidia and missing bristles.

As previously hypothesized, a potential explanation for the link between mutant DVAP and the resultant phenotypes with relation to Sac1 is that this interacting protein is also captured by the formation of aggregates and as such is removed from its normal localisation. Another hypothesis is that the aggregation of VAP removes the ability to form a complex that is necessary for Sac1 to function, perhaps in particular affecting its localisation at the ER and as such causing a similar effect as the reduction in Sac1 levels by RNAi. Regardless of the mechanism, a decrease in Sac1 protein levels recapitulates some of the phenotypic hallmarks of the mutant DVAP model which makes this a very attractive gene for further study with regard to degenerative disease. The maintenance of the Sac1 interaction despite the disease mutations in VAP is the next line of investigation.

Chapter 7: Investigation of VAP-CERT interaction

7.1 VAP mutation affects interaction with CERT

Human VAP has been shown to interact with a group of proteins containing a FFAT motif and one such protein is the Ceramide transport protein, CERT, as described in chapter 1. Considering that VAPB is functionally interchangeable for DVAP and the SAC1 interaction is maintained between the two species I hypothesised that the *Drosophila* homologue for CERT will interact with DVAP. This was initially examined by co-localisation in cell culture using COS7 cells. The cDNA for *Drosophila* CERT was obtained from the DGRC and subcloned into the pCMV-c-Myc vector containing the sequence for the c-Myc tag upstream of the multiple cloning site which will produce an N-terminally tagged fusion protein expressed under the control of the cytomegalovirus promoter. The pCMV DVAP-Flag construct cloned previously was again used and antibodies against the c-Myc and Flag tags were used to analyse the expression pattern for these two proteins.

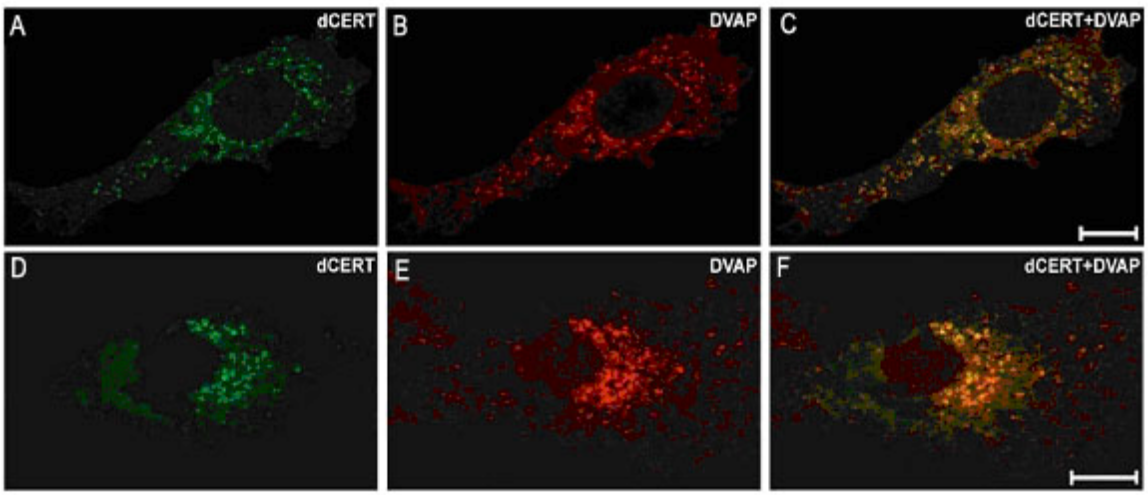


Figure 7.1 Colocalisation of DCERT and DVAP in COS7 cell
COS7 cells expressing DVAP-Flag and DCERT-Myc driven under the control of the cytomegalovirus promoter. Antibodies against Flag (red) and Myc (green) show extensive colocalisation which could indicate a physical interaction as present in other species.

Analysis of the localisation of DVAP and CERT when expressed in COS-7 cell culture shows a clear overlap which is suggestive of a physical interaction (figure 8.1). As

CERT is believed to interact with VAP via the FFAT domain it is likely that many other FFAT domain proteins shown previously to interact with VAPB will also show a conserved interaction with DVAP including Nir2 and OSBP. Considering the observed fragmentation of the ER following the transgenic expression of *DVAPT48I,* a loss of FFAT interaction would be fitting as it would recapitulate a DVAP null situation. To this end, Co IP's were carried out to test for the interaction of DCERT and DVAP and analyse the effect of the T48I mutation. A yeast two hybrid screen is currently underway to confirm the results from the Co-IP.

7.2 Co-IP of DVAP and DCERT shows an interaction that is lost following the T48I mutation

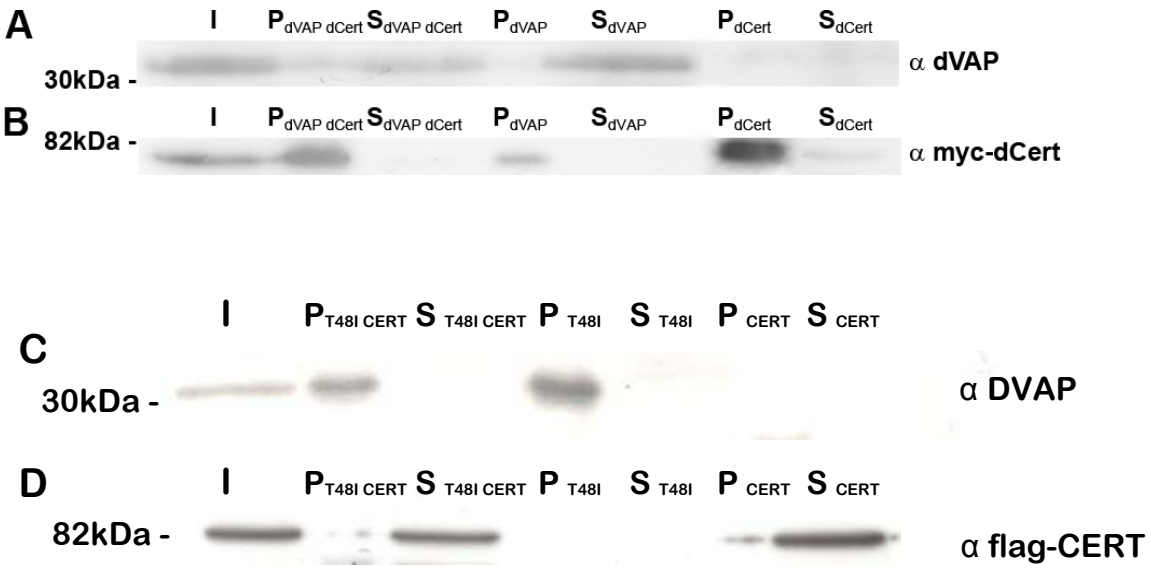


Figure 7.2 CoIP showing the loss of DVAP-DCERT interaction following the DVAPT48I mutation.
Western blots from a co-immunoprecipitation carried out with COS7 cells co-transfected with either wtDVAP (A and B) or mutant DVAPT48I (C and D) and DCERT. When testing the interaction of wt DVAP with DCERT, anti c-Myc agarose beads were used to bind the Myc tagged DCERT to act as bait for the any other interacting proteins. For DVAPT48I, a Myc tagged form of this mutant protein was used. The results heve been confirmed with the alternative arrangement of tagging.
A and B) Anti DVAP staining for the presence of wt DVAP in the input (I) and a faint but visible band in the test lane ($P_{dVAP\ dcert}$) confirms that wt DVAP can interact with DCERT. No band is present in the test lane of the single transformation controls. B) Anti-c-Myc staining for the presence of DCERT-c-Myc in the input (I) and attached to the beads ($P_{dVAP\ dcert}$ and P_{dcert}). There is no protein staining visible in the supernatant lane as all DCERT-c-Myc is attached to the beads.
C and D) Anti DVAP (GP33) shows a strong band in the test lane indicating that it has bound well to the beads but despite this there is no staining visible against flag in the test lane indicating that DCERT does not interact with mutant VAP. The T48I mutation disrupts the site necessary for the FFAT –VAP interact to occur.

The results from the Co-IP (Figure 7.2) confirm that the interaction between DCERT and VAP is lost following a mutation linked to ALS which gives a potential mechanism for the disease, especially considering that it probably means that none of the

interactions with the FFAT-containing proteins, Nir2, OSBP and CERT, will be possible. Removal of VAP was shown to lead to the relocalisation of FFAT containing proteins away from the ER into the cytoplasm and this might lead to an increase in Ceramide levels at the ER which has been shown to cause stress at this organelle (Peretti *et al.* 2008). The implications of the loss of this interaction could have serious effects on the cell and be a major part of the disease pathomechanism.

The potential breadth of knowledge to be gained by obtaining a better understanding of the role played by these few interacting proteins in the phenotypes seen following the transgenic expression of mutant VAP is very exciting. Only when all the pathways affected by the mutant proteins are realized can we truly hope to grasp what mechanisms lead to the degeneration observed both in our fly model and in human patients. For this reason a library scale yeast two hybrid was carried out in an attempt to find more interacting proteins.

Chapter 8: Library Scale yeast two-hybrid Screen.

8.1 Screening the Drosophila embryonic library

Following the successful results obtained in the small scale binary experiments the yeast GAL4 matchmaker 2 hybrid kit was used to carry out a library scale yeast two hybrid and screen a whole library of possible interactors. To be optimised at least $1x10^6$ clones should be screened and the transformation efficiency was calculated in order to determine the efficacy of the screen.

In total two screens were performed. The first, 3 different types of plates were used. Low stringency, medium and high. Colonies growing on the low stringency were collected and re-plated onto the high stringency plates. This extra step allows an initial phase of growth that increases the plasmid copy number and thereby increases the chance of finding proteins that may interact only weakly or transiently. In the second screen due to the high excess of interactors picked up from the first screen all transformed yeast was plated straight onto high stringency plates.

Screen 1.

Co-transformation efficiency: $\frac{\text{cfu x total suspension volume}}{\text{Vol. plated x dilution factor x ug DNA}}$

= 633cfu/ug

The ideal co-transformation efficiency for a library scale experiment is 10^3-10^4 so this first screen is a little low.

No. of independent clones screened: co transformation efficiency x ug library DNA
= 633 x 300 = 189,900 independent clones have been screened. Ideally at least 10^6 clones should be screened so the amount of DNA used must be adjusted accordingly.
Interactors from screen 1 are listed in the following table.

Gene number	Name	Role
CG3564	CHOp24	Protein transport Egg laying and embryogenesis
CG4264	Heat shock protein cognate 70	Molecular chaperone, ATPase
BT028801	-	Mitochondrial DNA
CG11949	Coracle	Gluatamate receptor anchoring
CG5185	Twin of M4	Antagonist of Notch signaling
CG3365	Drongo	ARF GTPase
CG6206	-	Alpha mannosidase activity
CG14206	Ribosomal protein s10b	Structural constituent of ribosome
CG4027	Actin 5c	Actin isoform. Component of cytoskeleton
CG11590	-	Cation transport acivity
CG17618	-	Unknown
CG8177	-	Anion exchange
CG7434	Ribosomal protein L22	structural constituent of ribosome
CG6444	-	unknown
CG17921	HMG Protein Z	DNA binding
CG5596	Myosin alkali light chain 1	Microfilament motor activity. ATPase activity
CG5014	Vap-33-1	Synaptic remodelling
CG8465	Lethal (1)G0222	unknown
CG4046	Ribosomal protein s16	Constituent of ribosome
CG5826	Peroxiredoxin 5037	Cell redox homeostasis
CG17903	Cytochrome C (proximal)	Electron carrier activity
CG10671	FIT2	Accumulation of lipid
CG5495	Thioredoxin-like	Cell redox homeostasis
CG32701	Lethal (l)G0320	Signal sequence binding
CG4005	Yorkie	Transcriptional co-activator, Negative regulator of apoptosis
CG18815	Acyl protein thioesterase1	Palmitoyl hydrolase activity
CG10718	nebbish	Chromatin binding kinesis-like protein
CG2163	Pabp2	mRNA binding
CG7533	Charybde	Negative regulator of growth. Head involution

CG10756	TBP associated factor 13	Transcription factor
CG40191	-	Water channel activity
CG8258	-	Protein folding
CG7326	Mek 1	Histone deacetylase
CG11164	-	unknown
CG3153	-	unknown
CG1782	Ubiquitin activating enzyme	Neuron remodeling, Protein modification
CG1783	dSLIP	Potassium channel regulator
CG7088	Bangles and beads	gliogenesis
CG3576	Longevity assurance gene 1	Transcription factor
CG5634	Distracted	unknown
CG7173	-	unknown
CG5226	-	Transporter activity
CG17521	Qm	Constituent of ribosome.
CG5703	NADH dehydrogenase	NAD binding. Electron transporter.
CG10364	Msb11	Unknown
CG10385	Msl-1	Chromatin binding. Dosage compensation.
CG6944	Lamin	Nervous system development, morphogenesis.
CG9568	-	Unknown
CG4038	-	rRNA pseudouridylation guide
CG2198	Amalgam	Antigen binding
CG5780	-	unknown
CG10203	X 16	Protein binding/mRNA splicing
CG6370	-	N-linked glycosylation

Table 8.1 Summary of interactions from Library Screen 1.
PGBKT DVAP was used as the bait protein to look for interactions in an embryonic library containing CDNA in the pACT2 vector. A number of the interacting partners have never been characterized and are known only as a gene number (CG). The activity in these cases is inferred from structural similarity with other known proteins. (Flybase)

Screen 2

Due to the large number of interactors found in the first screen the level of stringency was increased for the second screen so all colonies were plated straight onto medium or high stringency plates.

Co transformation efficiency = 4×10^3
Number of clones screened 3.7×10^6

The efficiency of this screen was within the desired parameters. The list of interactors is far fewer for screen two due to the increased stringency and also because a number of the clones found were repeated. Prior to sequencing, digestions are carried out in an attempt to avoid sending the same clones for sequencing but as was seen in the case of DVAP, in this second screen six different clones were sequenced and found to be DVAP cDNA of various lengths (all fragments of the C-terminus and often missing the MSP domain).

Gene number	Name	Role
CG5014	VAP-33-1	NMJ development/synaptic remodeling
CG4264	HSC 70-4	Molecular chaperone
CG3541	Pio	Tracheal development
CG6198	CHORD	Unknown
CG1516	-	Pyruvate Carboxylase activity/ Gluconeogenesis
CG32666	-	Protein threonine/serone kinase activity
CG5899	-	ATP-dependent DNA helicase activity
CG5146	-	Unknown

Table 8.2 Summary of interactions from Library Screen 2.
PGBKT DVAP was used as the bait protein to look for interactions in an embryonic library containing CDNA in the pACT2 vector.

Potentially interesting interactors were considered on a basis of previous characterization and tools available. A known role in cell morphology, neurodegeneration or any phenotypic changes possibly relating to ALS caused the following proteins to be picked as candidates for further investigation.

8.2 Interesting Interactions

1. Coracle

Table 1 - Coracle is the *Drosophila* homologue of the human erythrocyte protein 4.1. Originally characterized for its role at the septate junction where they link transmembrane proteins to the spectrin-actin- cytoskeleton (Fehon *et al.* 1994). The homologue in *Drosophila* has since been shown to be required for the localisation of Type A glutamate receptors by anchoring them to the actin cytoskelton in the postsynaptic bouton (Chen *et al.* 2005). The cDNA retrieved from the screen only contained the last 50 base pairs at the C-terminal of the coracle gene and the rest of the 3' UTR. The C-terminus is the domain that interacts with the glutamate subunits so this is still a feasible interaction. Unfortunately however, reproducing the interaction with the full length cDNA obtained from the *Drosophila* Genomics Resource Centre (DGRC) has not been possible.

2. Pio

Table 2 - Pio was identified for its mutant phenotype in *Drosophila* which includes a disintegrated tracheal system. Pio encodes a 462 aa long transmembrane protein with an N terminal signal perptide, a Zona Pellucida (ZP) domain, a putative furin cleavage site and a cytoplamic tail (Jazwinska *et al.* 2003). Pio is necessary for the formation of the tracheal system, possibly through the assembly of an apical extracellular matrix (aECM) (Paul and Beitel 2005). It is thought that it acts with another ZP domain protein known as Dumpy and that both proteins are cleaved at the furin site. This would leave the N-terminal portions containing the ZP domains free to interact and aid in the formation of

the aECM thereby providing the structure necessary to support the elongation and 'zipping up' of the endothelial cells of the tracheal system. Pio and Dumpy are also key to the formation of the inner layer of the aECM that forms as the outer layer of the wing. Flies that have had Pio or Dp disrupted have a blistered wing phenotype as the layers separate (Bokel *et al.* 2005). Again problems were encountered when trying to recreate the interaction with the full length cDNA received from the DGRC. A number of splice variants were constructed including removal of the transmembrane domain and the removal of the N terminal signaling peptide. Despite this there was still no interaction observed. See Chapter 8.3.

3. Yorkie

Table 1 - Yorkie is a transcriptional co-activator that positively regulates the growth of cells and is a downstream effector of Warts in the Salvador/warts/hippo tumor suppressor pathway. Yorkie interacts with the *Drosophila* consistency Scalloped (Sd) transcription factor which has the DNA binding capabilities that Yorkie does not and it promotes the nuclear localisation (Zhang *et al.* 2009). Warts phosphorylates Yorkie and inhibits the interaction with Sd and the nuclear localisation. The targets activated by Yorkie include *CycE* and *Diap* but there are likely to be many more that are in involved in organ growth (Pan 2007). The mammalian orthologue of Yorkie is Yes associated protein YAP and this is also the final target of a Hippo-like pathway that has been conserved from fly to man (Wang *et al.* 2009a). Further investigation of this interaction has not been continued but the interacting cDNA was complete for the open reading frame so it is likely to be a true interaction.

4. HSP 70

Table 1 and 2. An interaction found repeatedly is the heat shock cognate protein 70-4. Hsc70-4, a homologue of the human HSP70. Unlike other heat shock proteins Hsc70-4 is a constitutively expressed member of the HSP-70 family of chaperones which are necessary for the correct folding of new peptides, prevention of aggregates and the

refolding or degradation of damaged proteins (Broadley and Hartl 2009). Recently Hsc70-4 was found to be protective from hyperoxia induced neurodegeneration as it extended the lifespan of hyperoxia treated flies (Gruenewald *et al.* 2009). This interaction has not been verified but in the *Drosophila* model of ALS8, Hsp70 was found to be upregulated and relocalise to the aggregates. This may be a key player in the disease mechanism.

5. CHOp24

Table 1 - CHOp24 (CG3564) is one of 9 genes in the *Drosophila* p24 family that are homologous to the gene first discovered in Chinese hamster ovary cells (CHO) p24 (Stamnes *et al.* 1995; Blum *et al.* 1996). P24 proteins are type 1 integral membrane proteins located at the ER and Golgi and have been localised to both COPI- and COPII-vesicles (Wieland and Harter 1999). There are a number of hypothesized roles for the members of the P24 family including the formation of vesicles and regulating protein transport from the ER (Stamnes *et al.* 1995; Blum *et al.* 1996; Springer *et al.* 2000). The localisation of VAP at the ER means that an interaction with CHOp24 is feasible and the role of these two proteins may be linked.

6. FIT2

Table 1 - This *Drosopila* homologue of Fat inducing transcript 2 (FIT2) is a member of a highly conserved group of proteins initially described in mice for their role in fat storage (Kadereit *et al.* 2008). Located at the ER, overexpression of FIT in mice and cell culture has been shown to cause an accumulation of lipid droplets rich in triglycerides. We have preliminary data indicating that DVAP is also located at fat droplets in *Drosophila* (data not shown) and, in cojunction with the localisation at the ER, this could be a very interesting interaction. ALS patients often present with hyperlipidemia which also provides a link between this gene and the disease (Dupuis *et al.* 2008; Kadereit *et al.* 2008).

8.3 Interaction of DVAP with full length Piopio

The interacting cDNA of Pio consisted of the final 2 and a half exons of about 720 base pairs in length. This would be translated as a peptide of 240 amino acids in length (just over half of the full length protein of 462aa) and consisting of the transmembrane domain and half of the ZP domain.

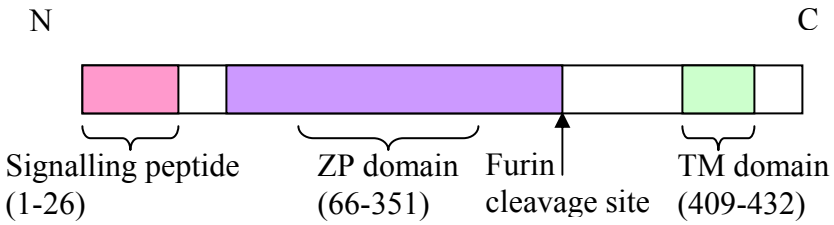


Figure 8.1 Dagramatic representation of Pio
Pio has the 3 different domains, N-terminal signal perptide, a ZP domain and a C-terminal TM domain. There is also a putative furin cleavage site. The C terminal protein sequence would be positioned within the cytosol of a cell and the N terminal outside in the extra cellular matrix.

When the interaction was tested with the full length protein, clone GH04558 from the DGRC, no interaction could be found with DVAP. It was hypothesized that the interaction could be interfered by the presence of the N-terminal signaling peptide, antagonizing the localisation to the nucleus so a clone was constructed missing the first 30 amino acids of the protein. It was also possible that it is the transmembrane domain that interacts with VAP and because the protein is normally cleaved at the furin site, when the whole protein is present it is cleaved and only the N terminus will remain attached to the GAL4 activation domain. This would mean that any interaction of the C terminus with DVAP would not be seen as an activation of reporter genes. For this reason a construct was also made with just the C terminal amino acids of Pio in the pGADT7 vector. Unfortunately neither of the shortened Pio constructs showed any interaction with DVAP.

Chapter 9: Discussion

9.1 Overview

Amyotrophic Lateral Sclerosis is a devastating disease of which only a limited understanding of the causes and mechanisms is known. The majority of cases appear to be sporadic but a few have known genetic causes. Much of the current research is focused on these few genetically linked cases and aimed at trying to get a clearer understanding of the role played by the mutated protein in the ensuing degeneration and progression of the disease. It is reasonable to believe that the pathways affected in the sporadic and genetic cases are similar if not identical and therefore any advancement made in these rare genetic forms is greatly beneficial to all. *VAPB* is a recent addition to the group of genes linked to ALS and as such, current research investigating the function of this protein has the potential to greatly increase our knowledge and understanding of ALS (Nishimura *et al.* 2004a). The aim of this project was to get an insight into the biochemical properties of VAP proteins and look at the complexes formed by the wild type protein to see which interactions are affected by the disease causing P56S mutation and the newly identified T46I mutation. Using a variety of model systems the effect of these two mutations has been examined and compared. The implication of all the results and the relevance to current published research will be discussed.

9.2 DVAPT48I recapitulates the phenotypic hallmarks of the DVAPP58S model of ALS

The first task was to examine the effect of the newly discovered T46I mutation on VAP proteins and compare the resulting phenotype following expression of this mutant protein with that of the original P56S mutation. The results from a number of experiments examining the *Drosophila* protein carrying the equivalent mutation showed that, consistent with *DVAPP58S*, the pan-neural expression of *DVAPT48I* leads to the formation of aggregates both along the nerve axon and in the cell body, see figure 3.2 (Chai *et al.* 2008) and figure 3.3. These aggregates are composed of both wt and mutant

protein as has been confirmed for the composition of P56S/P58S induced aggregates (Tsuda *et al.* 2008). Again in agreement with previous experiments the expression of *DVAPT48I* in the *Drosophila* eye caused a degenerative phenotype (figure 3.7) and the similarity in this range of mutant phenotypes indicates a similar pathomechanism for these two mutations. As more unrelated families suffering from ALS are found to carry a mutant VAP allele the justification for researching VAP proteins in relation to this disease increases. Although currently only a few cases of ALS have been attributed to VAP it is likely that many more will be found, perhaps in the upstream regulatory sequences that could disrupt the proper translation of VAP (Scheper *et al.* 2007). Such regions are rarely analysed during large scale screening of ALS patients (Conforti *et al.* 2006) so these mutations have yet to be found. With the identification of another VAP mutation linked to ALS and the lack of progress in the SOD1 mutant models, hopefully more resources will be put into the discovery of more VAP mutations in ALS patients.

9.3 Unfolded proteins and ER Stress

A significant finding in our model of ALS, following the over expression of *DVAPT48I* either pan neurally or in the muscle, is the fragmentation of the ER and the upregulation of HSP70 (see figures 3.5 and 3.6). Under normal conditions, proteins destined for secretory pathways are translated at the ribosomes of the rough ER and are moved into the ER lumen where they undergo proper folding and post translational modifications before translocating to the Golgi (Bukau *et al.* 2006; Szegezdi *et al.* 2006). ER chaperones are required for the correct folding and organisation of protein subunits and the internal climate of the ER is carefully maintained in order to prevent mis-folding and the formation of aggregates (Boot-Handford and Briggs; Bukau *et al.* 2006; Szegezdi *et al.* 2006). However, when mis-folding occurs, chaperones are in place to aid the correct refolding or if necessary, the removal of proteins to ER associated degradation (ERAD) by way of the 26S proteasome (Bukau *et al.* 2006). The careful environmental control necessary in the ER means that it is vulnerable to a number of environmental insults

including energy deprivation, altered Calcium levels, redox status, gene mutations, elevated protein traffic and altered post translational modifications (Szegezdi *et al.* 2006). In response to the accumulation of misfolded proteins (ER stress) the unfolded protein response (UPR) is activated which initiates a cascade of events designed to regain control of the system. The three ER stress sensors are Inositol requiring enzyme 1 (IRE1), Protein kinase-like ER kinase (PERK) and Activating transcription factor 6 (ATF6) (Boot-Handford and Briggs; Malhotra and Kaufman 2007). Together these proteins bring about a reduction in protein synthesis, an increase in chaperone levels and an increase in transcription of genes involved in ERAD. This all helps bring about the removal of the accumulated unfolded proteins but if in cases of an inability to solve the defect, prolonged UPR induces programmed cell death (apoptosis). The mutation in VAP and the formation of aggregates is understandably associated with an increased HSP70 level and fragmentation of the ER. The impact that the sequestering of wt VAP has on its interacting proteins must also impact upon the ER as an increase in Ceramide levels in the ER will disrupt the homeostatis of this organelle also (Wang *et al.* 2009b). The upregulation of HSP70 has been shown in a number of disease models to delay onset of disease symptoms by stabilizing mutated proteins and allowing some wt function (Mayer and Bukau 2005). As part of the aging process however, the ability of a cell to respond to ER stress is reduced and this might explain the late age of onset in a number of neurodegenerative diseases (Calderwood *et al.* 2009). If ER stress has a major role in such diseases, the age-related decline in UPR will lead to cells being overwhelmed by aggregating proteins and any other environmental insults on the ER bringing about cell death.

The reasoning behind the aggregation seen in our VAP model of ALS is possibly due to an increased propensity of the mutant protein to dimerise, especially with the wild type protein. We utilized the ability of VAP to homodimerise in an attempt to understand how the mutation affects the biochemical properties of both the *Drosophila* and human proteins by yeast two-hybrid.

9.4 Yeast two hybrid binary interactions

9.4.1 Homodimerisation of VAP

By yeast two-hybrid I have confirmed that DVAP has the ability to homodimerize and can interact both with wild type and mutant DVAP (figure 4.1). Homodimerisation is likely to be a necessary action for this protein to function and in agreement with this I and others have shown that the human and other mammalian proteins can also form dimers (Kanekura *et al.* 2006; Teuling *et al.* 2007). Previous studies have yet to find the yeast homologue Scs2p in dimeric form so this is perhaps an evolutionary advancement in the protein and a comparison of structural composition can give a clue as to the domains responsible for this interaction, as discussed below (Loewen and Levine 2005). The ability of the mutant proteins to homodimerise fits with theories about the implication of the mutation as rather than being a loss of function it is likely to cause the protein to gain a new function or be a hypermorphic allele. The current hypothesis adopted by our lab is that the mutation increases the strength of interaction, in particular with the wt protein and brings this into aggregates, thereby removing DVAP from its normal localisation. This mode of action is similar to a dominant negative mutation as despite the mutant protein retaining some of its wt functions, its presence disrupts the cells by decreasing the activity of the wt DVAP.

Following the creation of our *Drosophila* model for ALS using DVAPP58S a number of groups have supported our hypothesis and present a dominant negative role (Teuling *et al.* 2007; Ratnaparkhi *et al.* 2008; Tsuda *et al.* 2008). All report the sequestering of wt VAP into aggregates in the presence of the mutant protein. Perhaps the pioneering research into the implication of the disease mutation was reported prior to our model in 2006 by a group looking at the affect of the mutation on the interaction of VAP proteins using cell culture and GST pull down (Kanekura *et al.* 2006). Their findings are consistent with those reported here as they too found that the VAPBP56S mutant protein retained its ability to be homodimerized and proposed that the P56S mutation enhances the propensity for this interaction as seen by an increase in signal by

GST pull down. In a truncated protein missing the transmembrane domain Kanekura *et al* report a weak interaction with a full length protein and this is greatly enhanced by the P56S mutation. No such interaction was seen in the truncated protein of my yeast two hybrid but this is perhaps due to the size of the region removed and the different system used as the pull-down reported here is only very slight when the wt protein is truncated (Kanekura *et al.* 2006). If the MSP domain has a slight ability to homodimerise it might affect the dimerisation of the whole protein when mutated and increase the propensity for the wt and mutant protein to interact. Of course the *C.elegans* major sperm protein dimerises to form the actin like structures that propel the sperm forward and some of this ability may be retained in the domain of VAP (Tarr and Scott 2005). Our data however implicates the C-terminal portion of the protein as being ultimately responsible for the homodimerization and the decrease in pull down in the wt VAPΔTM protein supports this (Kanekura *et al.* 2006).

A study in 2000 looked at various transmembrane domain motifs and found the sequence GxxxG to be particularly common with reference to the ability of a protein to homodimerise (Russ and Engelman 2000). Table 2 of the report by Russ and Engelman (2000) identifies various amino acid combinations which have a strong association with dimerisation and of particular interest is the sequence featuring the large side chain residues of Valine, Leucine and Isoleucine alongside Glycine as this is exactly what is present in the human transmembrane domain and similar to the *Drosophila.* In *Drosophila* the homologous first Glycine residue is replaced by a Serine and both these amino acids are small in size (Figure 9.1). Whether it is this slight difference of the motif between these two species that causes a lack of interaction or the inability for the two regions to join as a consequence of an overall incompatibility in size and shape of the proteins as a whole is something unanswered here but it is evident that this slight difference makes a big difference when modeling the disease.

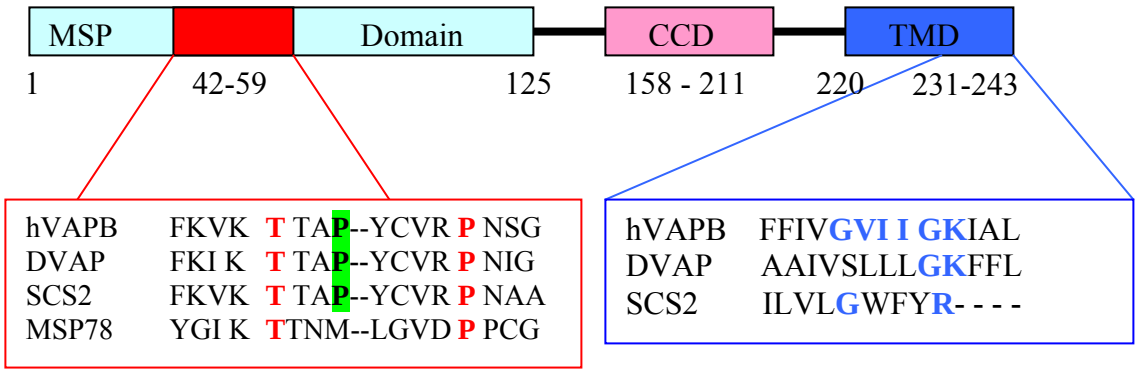


Figure 9.1 Primary organization of VAP
Diagram showing the primary organization of VAP homologues from human, *Drosophila,* and yeast. The MSP domain shows a high degree of conservation between species and compared to the original Major sperm protein of *C. elegans* (MSP78). Highlighted in red are the two current mutated residues, T46/48 and P56/58. The transmembrane domain contains the GxxxG motif in blue. Adapted from (Lev *et al.* 2008).

Many *Drosophila* models of disease have utilized the ability to express human genes in the fly and have proved invaluable in the progression of research in these diseases (Bonini and Fortini 2003; Cha *et al.* 2005; Greene *et al.* 2005). However the data presented here highlights a potential problem. If the possibility to express a mutation form of the endogenous *Drosophila* gene is available it may often be preferable as the foreign protein may not be able to recapitulate all the hallmarks of a disease due to an incompatibility within the system. Here we have previously shown that hVAP can rescue the null mutant which would indicate a full functional interchangeability but for such a disease model where an interaction with wt endogenous protein is so important, the human protein could not have been used in the model. Of course when the homologue in flies is unknown or perhaps has a very different primary sequence the use of human transgenes will always continue but it is a potential pitfall that should be acknowledged both in the fly world and other model organisms. Certainly in our case, the model expressing DVAPP58S has recapitulated the major hallmarks of ALS

remarkable well and provided a good example against which to test the new T48I mutation and any other mutations that may later be found.

In support of a growing amount of research, we find that the two mutations do not negatively interfere with the interaction of VAP with itself (figure 4.1). It is possible that the interaction is actually strengthened which may be tested in the future by methods such as GST pull down that provides results that can be quantitatively measured. However, perhaps more importantly is the effect of the mutations on the interaction with other proteins. Initially in agreement with the finding that DVAPP58S can rescue the null DVAP mutant fly was the finding that mutant VAP can still interact with the lipid phosphatase Sac1 (Figure 5.1). The C terminus of DVAP retains this interaction which is perhaps expected if the necessary domain for interaction is away from the site of mutation in the MSP domain. An understanding of the domains involved in various interactions with respect to the position of the mutated residues will give us some idea as to the impact the mutation should have on the interactions the protein forms.

9.5 Implication of VAP mutation on the function of Sac1

The exact domain of DVAP that interacts with Sac1 is currently being investigated by further yeast two hybrid studies. As described previously however, the potential effect of the maintenance of interaction following aggregation is of two categories both leading to a similar conclusion. Firstly Sac1 might be sequestered into the aggregates and removed from its site of action. Secondly, due to the removal of VAP away from the ER despite the ability to bind to VAP, the spatial barrier will mean that Sac1 will lose its binding partner and as such will have a reduction in function as a consequence of the loss of wt VAP. Both these scenarios will have a similar effect as both might be likened to a Sac1 loss of function and only by understanding the role of Sac1 can the potential effect of this on the cell be fully appreciated. Co-staining of cells expressing mutant VAP and Sac1 will need to be undertaken in order to examine the localisation of Sac1 in

mutant cells. An antibody is currently being created in order to look at the localisation of endogenous Sac1 in wt and mutant cells.

9.5.1 Current research of the role of Sac1

Sac 1 is a lipid phosphatase that dephosphorylates Phosphoinositide Phosphates (PIPs) to produce PIs that have been implicated in various roles including cytoskeletal organization, vesicle trafficking and apoptosis (Liu *et al.* 2008a). In *Drosophila* Sac1 plays a key role in dorsal closure (DC) and is a negative regulator of JNK activity (Wei *et al.* 2003a). The JNK MAPK cascade in *Drosophila* is necessary for the assembly of actomysin contractile apparatus and controls cell shape change and the transcription of a number of genes including members of the transforming growth factor (TGF_{β}) family (Noselli and Agnes 1999). Null mutations in *Drospohila* Sac1 results in defects in cell shape change with a premature morphogenesis of amnioserosa cells during DC (Wei *et al.* 2003a). In rats the activation of the JNK cascade has been linked to a decrease in glutamate release and an impairment of Long Term Potentiation (LTP) as well as cell death in the hippocampus (Minogue *et al.* 2003). JNK activation is believed to be the downstream effector of Aβ in rat models of Alzheimers disease and degenerating neurons of patients have been found to have increased JNK activation. As a negative regulator of JNK activity in flies, Sac1 may have a similar role in mammals. The mutation in VAP may affect the ability of Sac1 to regulate the JNK cascade and this is a potential explanation for many of the morphological changes witnessed at the neuromuscular junction and it may be causative for the degeneration of motor neurons. Results described earlier have shown that reduction in Sac1 causes an increase in the size of the synaptic boutons and also causes a degenerative phenotype in the *Drosophila* eye (figure 7.5).

Sac1 directly affects the formation of PIPs and investigations into the function of Sac1 in yeast have shown that Sac1 primarily metabolizes PI(4)Ps that are synthesized by Stt4P, a PI-4-kinase. A study by Foti *et al* demonstrated that the regulation of this

pool of PI(4)P is necessary for vacuole morphology, actin cytoskeleton organization and regulation of neutral lipid storage (Foti *et al.* 2001). Rapid inactivation of Sac1 in yeast is accompanied by dramatic changes in vacuole shape and a null *Sac1* mutation partially rescues the defects in actin cytoskeleton associated with a *Stt4P* null mutant. Stt4 was identified as a member of the Scs2p complex adding a further link between VAP proteins and the production of PIPs (Gavin *et al.* 2002). Another study found that cellular membrane homeostasis is coupled with the compartmentalization of individual PI(4)P-dependent processes and a deficiency in site specific regulation of PI(4)P due to a mutation in Sac1 induced defects in endosomal trafficking (Tahirovic *et al.* 2005). A number of studies have looked at the role played by PIPs in exocytosis in mammalian neurons and there is evidence that they are necessary for priming of vesicles (Wiedemann *et al.* 1998). In mice the loss of Sac1 causes preimplantation lethality(Liu *et al.* 2008a). In order to study the mammalian function of Sac1 and the cause of this lethality, Liu *et al* moved into HeLa cell culture and found that the knock down of Sac1 by RNAi caused a severe disruption of the Golgi and mitotic spindle which leads to cell cycle arrest. Whether it is a disruption of the regulation of the PI(4)P pool at the Golgi that is the ultimate cause of the change in its organization is a matter still to be confirmed.

In yeast, *Sec14* encodes a phosphatidylinositol transfer protein (PITP) that is required for the formation of Golgi-derived vesicles and a mutation in Sac1 acted as a suppressor of a *Sec14* mutant phenotype (Hama *et al.* 1999). This is particularly interesting considering that the mouse mutant, *Vibrator,* which has a reduced expression of PITPα, develops a progressive whole body action tremor with selective degeneration of neurons of the spinal cord, brain stem and dorsal root ganglia (Hamilton *et al.* 1997). It is not known if mammalian Sac1 also has a genetic interaction with PITP but it was found that expression of either of the rat homologues of Sec14 (PITPα or β) could rescue the mutant phenotype associated with a knock out of *Sec14* in yeast (Nemoto *et al.* 2000). They also demonstrated that rat Sac1 is the functional homologue of yeast Sac1p and as such it is very likely that Sac1 is functionally linked to the role of PITPs in mammals.

In yeast there is believed to be a number of pools of PIPs which are independent of one another. One particular pool is at the ER and this is controlled particularly by Stt4p and Sac1 (Konrad *et al.* 2002; Tahirovic *et al.* 2005; Faulhammer *et al.* 2007; Knodler *et al.* 2008). VAP's localisation at the ER may be key to this function and its removal may have serious implications on the role of Sac1 here. Also, removal of Sac1 might have other affects on the ER and the disintegration of this organelle will greatly affect a number of proteins with roles linked here. Considering that mutations in *Fig4*, another lipid phosphatase involved in the removal of phosphate groups from the 5th head group of PI(3,5)Ps, have been recently linked to cases of ALS there is a strong case for the further study of these lipid molecules with reference to ALS and other neurodegenerative disease (Chow *et al.* 2007).

9.6 DVAPT48I no longer interacts with the FFAT domain protein CERT

Research has shown that VAP is necessary for the anchoring of a number of proteins to the ER (Amarilio *et al.* 2005; Kaiser *et al.* 2005; Kawano *et al.* 2006; Peretti *et al.* 2008). This is via an interaction of the FFAT motif contained within these proteins to a region of positive residues in the MSP domain of VAP. Such FFAT containing proteins include CERT, OSBP and Nir2. The crystal structure of the VAP-FFAT interaction has been solved and the residues necessary for the interaction mapped (Kaiser *et al.* 2005). The phenylalanine within the FFAT tract has extensive Van de Waals contacts with a number of VAP residues which create a positive pocket. Crucial residues for this are Met 89 and the sidechains of Lys 45, Thr47, Lys 87 and Lys 118. Another screen in yeast also found the equivalent residues to be necessary for the FFAT interaction (Kagiwada and Hashimoto 2007). In the rat Orp1 protein sequence, the Ala479 is also important interactor and binds VAP in a small hydrophobic pocket created by Val 44, Thr 46 and Val54 (Kaiser *et al.* 2005). This is particularly of note because it is the Thr46 that is mutated in the new ALS 8 patients. Both residues have hydrophobic side

chains but Isoleucine has an extra CH_2 which can be expected to disrupt the configuration of the pocket. To a similar extent with regard to the previous mutation, the change of the large cyclic structure of a Proline residue for a small polar Serine at position 56 in the sequence so close to these important residues is also likely to interrupt the binding domain. These hypotheses were found to be true, at least by Co-IP, as no interaction was found for either DVAPP58S or DVAPT48I with DCERT whereas wt DVAP showed good co-immunoprecipitation (figure 8.2). In agreement with our data, Teuling *et al* found that following the P56S mutation the interaction between VAPB and Nir2 or a number of ORP proteins were lost as seen by pull down assays and mass spectrometry (Teuling *et al.* 2007). Again a full understanding of the role of these proteins is necessary for gaining an insight into the potential problems that such a loss of interaction would have in relation to the disease and our model. Luckily, experiments have already been carried out to investigate the effect of a knock down of VAP on the localisation of these proteins.

9.6.1 Role of CERT, Nir2 and OSBP

A study by Perretti *et al* found that upon removal of VAP, the localisation of CERT, Nir2 and OSBP at both the ER and the Golgi was affected. These proteins, involved in the synthesis and trafficking of lipids, shuttle between these two organelles and the loss of Golgi localisation was attributed to the loss of PI(4)P which is necessary for anchoring the proteins via their PH domains (Peretti *et al.* 2008). This is important considering that Sac1 is a lipid phosphatase and removes the phosphate group from PI(4)P. Disruption of Sac1 is likely to affect the pool of PI(4)Ps at the Golgi and could in turn affect the localisation of these proteins. Over expression of VAP in the presence of Nir2 has been shown to affect the arrangement of the ER and is was suggested that in some cases, VAP proteins act more as a receptor with the interaction inducing a conformational change that in turn affects the organization and positioning of the ER. (Amarilio *et al.* 2005) The position of the ER is linked to the microtubule network and

previously VAP has been shown to affect the organization and stability of the microtubules (Pennetta *et al.* 2002). Amarilio *et al* find that the presence of Nir3 is necessary for the formation of microtubule bundles and it is hypothesized that this could act as the bridge between VAP and the microtubules that has been missing up until now. OSBP and CERT also have a strong link with VAP and the role at the ER. In response to oxysterol binding or the depletion of cholesterol, OSBP translocates with CERT to the Golgi where upon SM synthesis is stimulated (Perry and Ridgway 2006). OSBP was also found to be necessary for the interaction of CERT with VAP at the ER and it was hypothesized that both proteins could interact in a heterodimer along with the homodimer of VAP. When examining the FFAT interaction, it was proposed by Kaiser *et al* that interactions formed with VAP may occur in a 2:2 complex (Kaiser *et al.* 2005). If OSBP and CERT interact they may together bind the VAP homodimer and the interaction of VAP and CERT is increased when sterol levels are low as sensed by OSBP (Perry and Ridgway 2006).

The production of Sphingomyelin and DAG is tightly regulated at the Golgi by a number of negative feedback loops. DAG is the substrate of the CDP Choline pathways which synthesizes PC at the Golgi membrane (Hanada *et al.* 2009). Transfer of PI to the Golgi from the ER by Nir2 is coupled with the removal of PC to the ER. This allows for the production of PI4P at the Golgi which provides a target for the PH domain of CERT and OSBP (Peretti *et al.* 2008). OSBP activates CERT and leads to the transfer of Ceramide from ER to the Golgi and into the hands of SM synthase which produces SM and DAG (Perry and Ridgway 2006). VAP is a key targeting protein in this system as it is necessary for the binding of Nir2 at the ER-Golgi membrane contact sites and upon its depletion, a reduction in the levels of PI4P, SM and DAG at the Golgi were all noted which can be attributed to a lack of binding of these three FFAT containing proteins (Amarilio *et al.* 2005; Perry and Ridgway 2006; Peretti *et al.* 2008). This has an affect on the structure and function of the Golgi and causes a decrease in protein transport to the plasma membrane, endosome, lysosome and ER (Peretti *et al.* 2008).

In our model little has been noted of the Golgi so this is an area of future research but the ER is certainly affected and looking at the roles of these proteins it is

possible to see how a removal of VAP might also affect the ER. One aspect will be the accumulation of Ceramide at the ER and this has been shown both in *Drosophila* and mice. Mice that have been bred as double mutants for CERT are embryonic lethal and die at E11.5 (Wang *et al.* 2009b). Examination of the ER and mitochondria show both these organelles to be under stress as inferred from the engorgement and vesiculation of the ER and engorgement of the mitochrondria accompanied by functional abnormalities. The total levels of ceramide remained unchanged as compared to control animals but the levels within the ER and mitochondria were increased suggesting an inability of the Ceramide to be transported away from the ER due to a loss of CERT. The mitochondria are believed to receive ceramide directly from the ER via close contact sites through a mechanism apparently independent of CERT. The increased level within these two organelles puts them under severe stress as seen by the changes in physiology and function. Despite these problems the mice survive up until E11.5 at which point the cause of death is from a malformation of the cardiovascular system due to an interruption of the cell cycle and differentiation. Many downstream targets of the UPR are up regulated including eIF2-α which is involved in cell cycle arrest and protein translation following ER stress. Chronic ER stress in these animals is believed to be the cause of the cellular disruptions which leads to the embryonic death (Wang *et al.* 2009b).

In *Drosophila* the knock out of *DCERT* has a severe effect on lifespan and males live between 10-20 days, females up to 30 days. Ceramide Phosphoethanolamine (CPE) is the *Drosophila* analogue of SM and in these mutant flies both the levels of CPE and Ceramide are decreased by about 70% (Rao *et al.* 2007). This leads to a decrease in membrane density causing an increase in fluidity and a susceptibility to Oxygen free radicals which react with the membrane lipids to produce lipid peroxidation products. The mitochondria is also affected and believed to be under chronic stress which compromises function as seen by a reduction in cytochrome C Oxidase activity which is also evident in the mouse model (Rao *et al.* 2007; Wang *et al.* 2009b). Such data linked to a reduction in CERT could also be expected from a model in which VAP has been removed and as such the localisation of CERT, Nir2 and OSBP affected. The changes in

the ER may be both as a direct consequence of the increase in Ceramide levels in this organelle and also as a result of the aggregation of VAP. Teuling *et al* report that the aggregates formed by mutant VAP consist of ER-derived tubular structures and the fragmentation seen in our data may indicate a similar process is occurring here (see figure 3.5 and 3.6) (Teuling *et al.* 2007) In the mouse CERT KO model a number of pathways were down regulated in response to the ER stress and one such pathway was the JNK cascade which has been mentioned earlier in respect of the role of Sac1 (Wei *et al.* 2003a; Wang *et al.* 2009b). Sac1 is a negative regulator of the JNK cascade and it might be imagined that following the removal of VAP, the cellular ability to compensate for the increase in Ceramide might further be compromised by a lack of other regulatory pathways also linked into the role of VAP.

The role of lipid molecules may be further instigated in the story of ALS by the finding that diets high in lipid content are beneficial in ALS and delay disease progression (Dupuis *et al.* 2004; Dupuis *et al.* 2008). SOD1 mouse models of ALS showed an increased energy expenditure causing the mice to be leaner than their wt littermates despite a normal rate of food consumption. Feeding a fat enriched, high energy diet to these mice allowed them to gain weight and actually reversed the expression of some denervation markers suggesting a delay in disease progression (Dupuis *et al.* 2004). In humans, two thirds of ALS patients have been shown to have a stable hyper-metabolism which is linked to survival and a number of patients also present with hyperlipidemia despite having a lean physique. Such patients tend to live longer (Dupuis *et al.* 2008). Exactly why this might be the case is unknown but is possibly linked to the increased energy expenditure required for regeneration. *Drosophila* is a good system for modeling changes in metabolism as a number of the key regulatory pathways are conserved. The *Drosophila* fat body can be considered as the equivalent to adipose tissue in humans meanwhile there are the oenocytes that have been described as hepatocyte-like cells (Bharucha 2009). A study investigating the role of the Ceramide synthase gene named *Schlank* found that a reduction in the expression of this gene caused a reduction in the fat storage of the fat body (Bauer *et al.* 2009). Similarly over expression caused an increase in triacylglyceride (TAG), DAG and fatty acids

(FA). It could be considered that fat storage will be affected in our fly model if the Ceramide transport is disrupted by the loss of CERT localisation to the ER and if so this is another key hallmark that can be investigated in our model. The necessity of VAP in the production of Ceramide, SM and other lipid molecules in the cell puts this protein right in the middle of this important field of research.

9.7 The role of VAP as an Eph receptor ligand

The extensive knowledge to be gained through a fuller understanding of the interaction of VAP with these lipid regulating proteins is just one part of the jigsaw puzzle that is ALS. The afore mentioned interaction with Eph receptors as demonstrated by Tsuda *et al* is also of huge importance as these Tyrosine Kinase receptors and the multiple downstream pathways which they regulate are linked to a number of aspects of synaptogenesis, synaptic plasticity and axonal guidance as well as angiogenesis and neural crest migration (Adams *et al.* 2001; Klein 2001; Dearborn *et al.* 2002; Tsuda *et al.* 2008). In vertebrates there are two types of Ephrin ligand. Ephrin As are membrane bound and anchored by glycosylphosphatidylinositol (GPI) and they are the ligand for Eph A receptors. Ephrin B are phosphorylated on cytoplasmic tyrosyls following the binding of Eph B receptors (Chin-Sang *et al.* 1999). The invertebrate *C. elegans* has only one receptor Vab-1 and four ligands. Vab2/EFN-1 is a GPI-anchored ephrin similar to the type A of vertebrates but in sequence they are similar to Ephrin B suggesting that this is a common ancestor for the two vertebrate ligands (George *et al.* 1998; Chin-Sang *et al.* 1999). Tsuda *et al* believe the MSP domain of VAP is cleaved and released from cells and from evidence in *C. elegans* it is thought that this domain might act on Eph receptors in the surrounding environment. Considering that our disease model in *Drosophila* has shown a re-organisation of the glutamate receptors postsynaptically and a change in the morphology of the synapse, such a role of the MSP domain is very exciting. The Eph receptors activate a number of down stream pathways which gives them control of an array of cellular mechanisms at the synapse and affect pre and

postsynaptically by forward signaling and reverse signaling back to the pre synaptic terminal from which they were released (Pasquale 2008; Lai and Ip 2009). They have been shown to play a key role in the clustering of AMPA and NMDA receptors in mammalian cells and are necessary for the motility of filopodia which precedes spine formation during the development of synaptic contacts (Kayser *et al.* 2008; Lai and Ip 2009). Signals sent between pre- and post- synaptic membranes is vitally important for the correct formation of connections and by signaling between neurons a communication network is accomplished that allows for the precise pattern of neuronal circuitry seen in the nervous system. If the MSP domain of VAP is cleaved and secreted it can be imagined how this might impact upon the formation and homeostasis the synapse (Tsuda *et al.* 2008). If this signal is removed due to the disease mutation causing aggregation of the full length protein and an inability to cleave and release the MSP domain then this homeostatic signal would be lost.

Synapses have a remarkable ability to compensate for the loss of neighbouring synapses by the sprouting and formation of new connections (Gillingwater *et al.* 2006). ALS is a late onset disease with symptoms not presenting until past the age of 50 in the majority of cases. Such a pattern of disease might be attributed to a decline in the ability of the nervous system to compensate for stress put on the system. A general age-related decline in a number of homeostatic and stress response mechanisms such as the UPR and synaptic sprouting may be underlying the late onset occurance of a number of degenerative diseases. Here it could be hypothesized that only after many years does the cell become overwhelmed and unable to cope with the aggregation of VAP leading to a greater decline in the available wt VAP and a decrease in Eph signaling coupled with a build up of Ceramide in the ER. Perhaps it is the stress caused on this organelle and others such as the mitochondria and Golgi that overloads the system that has been under strain for many years previously and a fairly rapid decline is seen. The apparent multiple roles of VAP proteins means that once the quality control system of the cell starts to fail, multiple downstream targets are affected by the loss of VAP and the ER is put under further stress so that a fairly rapid deteriorating cycle might occur.

This all makes for an interesting hypothesis but it is unlikely that this is the whole story and for this reason I carried out the library scale yeast two hybrid.

9.8 Library Scale Yeast two Hybrid Screen

There are a number of interesting findings in the screen and overall the screen was working well as I repeatedly pulled out VAP which shows that I was able to find a positive interactor. However there was a continual problem of finding interactors that were in non coding regions of a gene. After a number of repeat platings of a positive colony on high stringency AHLT plates, in an attempt to select only for the interacting clone, and then the process of extracting the DNA and amplifying it in bacteria and sending for sequencing often the sequence received contained only 3'UTR. All the results in table 9.1 and 9.2 contain ORF but not always the whole protein and as such, they may be anomalies. The kit we used was the MATCHMAKER GAL4 Two-Hybrid System 3 from Clontech with the *Drosophila melanogaster* embryo MATCHMAKER cDNA library as we wanted to use DVAP as the bait protein. The average length of cDNA in the library was known to be 1.4kb ranging between 0.5-3kb which means that a lot of the cDNA is truncated which is a problem as in addition to getting false positives, we will also miss interactions that require the full length protein such as Sac1. There is now a human library available in which new SMART^{TM} cDNA synthesis technology has been used to obtain longer lengths of cDNA and this will hopefully prove to be better if another yeast two hybrid screen were to be undertaken (Clontech).

Despite the problems, a number of the results look promising and will be investigated in due course. Proteins will be selected on the basis of length of the interacting cDNA, the knowledge about the interactor and the available tools including antibodies and *Drosophila* stocks and finally on the link between said protein and roles in which VAP is believed to play. A genetic enhancer suppressor screen for the degenerative eye phenotype of VAP was carried out by Andrea Chai and any overlap in results will greatly increase the likelihood of a meaningful interactor. Already one such

interactor is Bangles and Beads which is believed to be involved in gliogenesis due to the pattern of expression but there is little further information known (www.flybase.com). A known co-localisation of proteins with VAP will also make an interaction more trustworthy, an example being FIT2, an ER localised protein that is part of a conserved family involved in the accumulation of lipid droplets (Kadereit *et al.* 2008). A number of proteins are being confirmed as co-localising with lipid droplets in *Drosophila* and we believe DVAP to be one of them (data not shown). Important tools will include stocks containing P-element insertions or mutations that disrupt the gene of interest, full length cDNA to enable the cloning of the gene into *Drosophila* transformtation vectors and antibodies against the protein. Such time saving tools would allow for the quick identification of important interactions and likewise the ability to quickly rule out those that are not involved in the phenotype of our VAP mutants.

Chapter 10: Future Prospectives

Chapter 10.1: Looking to the future

In order to advance our understanding of the disease pathomechanism of ALS and other neurodegenerative diseases it is vital that we dissect out the sequence of events that leads to the phenotypes observed. *Drosophila* is a perfect tool for looking for genetic interactors that affect the disease phenotype and we have a number of well characterised phenotypic markers including the degeneration of the eye, larval locomotion, aggregate formation and ER fragmentation (Chai *et al.* 2008). Already Andrea Chai has carried out a screen for genetic modifiers of the degenerative eye phenotype by using a kit of deficiency lines composed of flies carrying various P element insertions that disrupt various numbers of genes. Here I have carried out a library scale yeast two hybrid in an attempt to find genes involved in ALS by the direct interaction with DVAP and this has produced a long list of proteins possibly involved in the function of DVAP. Taking the list of interactions that resulted from the library yeast two-hybrid screen and transforming it into a comprehensive list of suitable targets for future study is the next step to be undertaken. By investigating the tools and knowledge available for each interactor a shortlist can be created that can then be used to target our attempts to uncover the pathways in which DVAP acts and the events that lead to degeneration. A few of the genes are discussed here but there are many genes that may be worth further investigation, including those that have yet to be characterised.

One of the first interacting proteins found in the screen was CHOp24. This is a member of the p24 family of proteins which are type 1 integral membrane proteins involved in intracellular trafficking between the ER and the Golgi (Carney and Bowen 2004). The expression pattern for CHOp24 during larval development up until adulthood is ubiquitous in all tissues examined and the sub cellular localisation is at the ER which makes this a plausible interaction (Boltz *et al.* 2007). An investigation into the feasibility of further researching this protein in relation to DVAP presents some promising data. CHOp24 (CG3564) is present on the X chromosome at position 4C7 and it is composed of three exons (Flybase). The Bloomington Stock Center holds two available stocks that have piggybac insertions within the 5' UTR of CHOp24 named

PBac(RB)CHOp24[e04526] and PBac(RB)CHOp24[e04567]. It is possible that these will disrupt the gene and provide an opportunity to study the function of this protein in *Drosophila.* There is also an RNAi line available from the Vienna *Drosophila* RNAi Centre (VDRC) termed w[1118]; P(GD3225)v7039. I believe it would be worthwhile obtaining these stocks and observing the resulting phenotype from the disruption of the gene. If results are promising, the creation of a *UAS-CHOp24* should be undertaken in order to look at the effect of over expression. It is likely that the various hallmarks of disease seen in our *Drosophila* model are due to a number of different downstream targets and it would be expected that the involvement of an interacting protein such as CHOp24 might be to contribute to subset of the phenotypic markers.

FIT2 (CG10671) is a promising interaction by way of its proposed involvement in lipid storage (Kadereit *et al.* 2008). Similarly to VAP it is located at the ER where it is believed to enhance the partitioning of trigylcerides into lipid droplets (Kadereit *et al.* 2008). *FIT2* is present on the third chromosome (3L) at position 64C7 and is composed of 2 exons (Flybase). There is unfortunately no P-element insertion within the open reading frame of this gene but there are two insertions available at the extreme 5' UTR. These may be useful as they may still interfere with the translation of the gene or, if not, they could possibly be jumped further into the gene by a phenomenon known as local hopping (Zhang and Spradling 1993). By crossing a P-element insertion line with a stable source of transposase the resulting mobilization of the P element occurs in the germline of females and this transposition tends to occur within integration sites nearby (0-150kb away) Unfortunately however there is some overlap of the 5'UTR of *FIT2* with 5'UTR of a neighboring gene *Aats-ala-m*. Jumping out the P-element may disrupt this gene so any phenotype observed will have to be rescued with the transgenic expression of wt *FIT2* to ensure that it is not due to the disruption of Aats-ala-m, a little known protein believed to have alanine-tRNA ligase activity (Flybase). There are also two RNAi stocks available w[1118]P(GD3580)v44435 and w[1118]; P(GD3580)v44433 that can be used to observe the effect of silencing *FIT2* expression.

Both FIT2 and CHOp24 would certainly warrant further investigation in view of the localisation at the ER, an organelle so central to the VAP story. Yorkie (Yki) on the

other hand is a transcription co activator that shuttles between the cytoplasm and the nucleus and rather than be selected for its localisation, it is its function as an anti-apoptotic factor that makes this protein a potentially interesting interactor (Kadereit *et al.* 2008; Wang *et al.* 2009a). VAP may interact with Yki in the cytoplasm and disruption of this pathway, especially the retention of Yorkie away from the nucleus in aggregates, could cause cell cycle arrest and induce apoptosis. There are a number of *Drosophila* P-element insertion lines for Yki (CG4005) including PBac(WH)yki^{f06249} from the Harvard stock centre and Mi(ET1)yki^{MB09079} available from the Bloomington stock centre. The gene is located on chromosome 2R, consisting of 5 exons and is free from overlap with other neighboring genes (Flybase). There are a number of *UAS-Yki* lines that have been created and are available from the Bloomington stock centre including *UAS-Yki-GFP* and there is also an RNAi line available from the VDRC W[1118];P(GD11187)v40497/TM3 Finally an antibody has been raised in rabbits against Yki (Oh and Irvine 2008) allowing the localisation to be observed i*n vivo* which will make it possible to check for the co localisation with VAP. The availability of so many tools makes for a very promising project and the immediate confirmation of the interaction with VAP by Co-IP, GST pull down or co-localisation should be undertaken.

As described in the results section 9.2 it was not possible to reproduce the interaction for DVAP with the full length cDNA for Coracle and PioPio. However it may be worth pursuing these interactors as the respective roles in glutamate receptor anchoring and tracheal system formation make these proteins very interesting (Chen *et al.* 2005; Paul and Beitel 2005). The tracheal system of *Drosophila* has been used as a tool for the study of angiogenesis and a link between angiogenesis and ALS has been found in patients (Storkebaum *et al.* 2004; Lambrechts *et al.* 2006). Piopio is on Chromosome 2R and there are two available insertions from the Bloomington stock centre located within the 5'UTR (Flybase), w1118; PBac{WH}piof04210 and w^{1118}; Mi{ET1}pio^{MB03570} that may themselves disrupt the gene or by jumping out by imprecise excision could lead to null mutants. Again there is also an RNAi stock w^{1118}; P{GD4824}v12877/TM3 that is available from VDRC. An antibody has been raised against Piopio (Jazwinska *et al.* 2003) and if a colocalisation with VAP can be

confirmed this would support the continuation of this project. Similarly an antibody is available against Coracle (Fehon *et al.* 1994) that can be used to check for a colocalistion with VAP prior to obtaining P element insertion lines that are available from Bloomington and Harvard Stock Centers and the VDRC. Despite the lack of interaction of the full length protein in yeast cells an interaction should be obtainable by Co-IP or GST pull down and this should be attempted.

From the library scale yeast two hybrid I had hoped to pick up OSBP as this is a well known interactor of VAP and CG1513, a *Drosophila* gene with high homology to the mammalian OSBP, was identified as a DVAP interactor in a genome wide yeast two-hybrid screen (Giot *et al.* 2003). Another *Drosophila* homolog is CG6708 which interacts with *farinelli*, a VAP homologue found only in the male testes (www.thebiogrid.com). The previous interaction with DVAP makes CG1513 a lucrative gene for further study but the genomic region is very complex as there are 15 exons and two other genes are present within the first two introns (Flybase). These two genes are CG30008 and CG12923 and although CG12923 is only expressed in the adult testes, CG30008 is ubiquitously expressed and believed to have a role in fatty acid synthesis (Szafer-Glusman *et al.* 2008). Any P elements or mutations within this region must be verified as causing phenotypes directly linked to the interuption of CG1513 by rescuing the phenotype with wt CG1513 cDNA. As it is, there is an insertion within the third exon from the 3' of CG1513 which falls within the putative Oxysterol binding domain (Levine and Munro 2001) so may significantly affect the function of the protein PBac CG1513^{c05643} (Minogue *et al.*). There are also some P elements inserted 1.4 kb downstream that could be jumped into the gene by local hopping transposition P(lacW)l(2)k06204^{k06204}. Previous attempts of obtaining full length cDNA from the BDGP were unsuccessful so this interaction has not been confirmed as yet but as it is so well conserved in other organisms it should warrant some further investigation if possible (Wyles *et al.* 2002; Wyles and Ridgway 2004). The interaction with OSBP occurs through the FFAT domain and the conservation of the DVAP-CERT interaction and the effect of the DVAPT48I mutation make this a very interesting interaction.

The increasing understanding that ALS is more than a disease of the motor neurons and actually affects a number of systems (multisystemic), means that the search for potential candidates involved in the disease pathomechanism widens. Only when a full knowledge of the pathomechanism of disease is gained and all the contributing factors have been realized can there be hope of substantial advancements towards a prevention or cure of this devastating disease.

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Appendix 1: ***hVAPB, the causative gene of a heterogeneous group of motor neuron diseases in humans, is functionally interchangeable with its Drosophila homologue DVAP-33A at the neuromuscular junction*****. Chai *et al.* 2008 Hum Mol Genet** 17**(2): 266-80.**